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AUTHOR – AUTEUR

Full Name of Author – Nom complet de l'auteur

GILBERTO FABRIS

Date of Birth – Date de naissance

NOV. 24 1956

Canadian Citizen – Citoyen canadien

☑ Yes / Oui ☐ No / Non

Country of Birth – Lieu de naissance

CANADA

Permanent Address – Résidence fixe

APT 7136
4501 - 37 ST. N.W.
CALGARY, ALTA
T3L 2J5

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University – Université

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Name of Supervisor – Nom du directeur de thèse

DR. C.D. JEWELL

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LOCALIZATION OF WESTERN EQUINE ENCEPHALITIS (WEE) VIRAL ANTIGEN IN SUCKLING MICE USING THE PEROXIDASE-ANTIPEROXIDASE TECHNIQUE

by

GILBERT FABRIS

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FUFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

IN

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Supervisor

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External Examiner

Date Aug 6/1985

ABSTRACT

This study was designed to localize Western equine encephalitis (WEE) viral antigen in the brain and visceral tissues of experimentally infected suckling mice. The peroxidase-antiperoxidase (PAP) technique was utilized to identify viral antigen and monitor the pathogenic process. Mice (1-3 day old) were inoculated either intracerebrally or intraperitoneally and subcutaneously. Suckling mice infected with WEE virus developed clinical symptoms of encephalitis 34-38 hours post-infection. Eastern equine encephalitis (EEE) virus, utilized as a cross-reactive control antigen in the PAP staining procedure, produced evidence of an acute encephalitis after 32-36 hours. The most notable gross pathological feature was the presence of congested blood vessels on the surface of the brain. A bland histopathological picture was observed in tissue sections taken from mice demonstrating clinical signs of encephalitis. A rare necrotic neuron was the only visible histopathological feature in the central nervous system (CNS). Similarly, visceral organs were normal histologically save for a mild pneumonitis and congestion of the liver in a few of the EEE virus infected animals.

Immunoperoxidase staining of brain tissue revealed sites of WEE viral antigen as dark brown granular deposits. In initial experiments extensive non-specific staining of the neuropil in the form of a muddy-brown colouration was observed but absorption of the primary reagent, mouse anti-WEE virus sera, with normal mouse brain tissue significantly reduced the level of background non-specificity in the neuropil. Trypsinization of the tissue sections, however did not greatly reduce the intensity of the background stain. Various interpolated experiments to reduce this non-specificity including varying the

duration and temperature of the incubation period of the primary reaction, treatment with imidazole, and switching of link reagents provided moderate improvement in the staining quality. Ultimately, a checkerboard titration revealed that mouse anti-WEE virus sera at a dilution of 1:1500 incubated with tissue sections at 4°C for 48 hours provided the best staining results.

An extensive distribution pattern of WEE viral antigen was observed in the brains of mice infected intracerebrally following staining by the PAP procedure. The CNS of mice infected intraperitoneally and subcutaneously exhibited a somewhat restricted distribution of viral antigen. The most severely affected areas of the neural tissue were the midbrain, pons, and medulla. The periventricular regions of the midbrain and pons, as well as thalamic nuclei, and portions of the hippocampus were acutely sensitive to this arbovirus invasion. Purkinje cells were often immunoreactive, whereas the choroid plexus and the substantia nigra were negative. Specific staining was effectively limited to the neurons and ependymal cells. Occasionally, glial cells appeared to contain antigenic markers for WEE virus.

WEE viral antigen was not detected in any of the visceral organs tested. However, evidence of viral replication was seen in the trigeminal ganglion and in the ganglion layer of the retina.

Positive staining of WEE virus was accomplished in 4 hours proving that the PAP technique can be used as a rapid diagnostic system for the identification of viral agents in clinical specimens. This investigation further substantiates the effectiveness of the PAP technique as a tool for the study of viral encephalitis.

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LIST OF ABBREVIATIONS

CTF	Colorado Tick Fever
DAB	Diaminobenzidine
EEE	Eastern Equine Encephalitis
ELISA	Enzyme Immunosorbent Assay
GAM	Goat anti-Mouse
GAR	Goat anti-Rabbit
HRP	Horseradish Peroxidase
IF	Immunofluorescent
IP	Immunoperoxidase
LD_{50}	50% Lethal Dose
M-PAP	Mouse Peroxidase-Antiperoxidase
M-WEE	Mouse anti-Western Equine Encephalitis
PAP	Peroxidase-Antiperoxidase
RAM	Rabbit anti-Mouse
R-WEE	Rabbit anti-Western Equine Encephalitis
SLE	St. Louis Encephalitis
WEE	Western Equine Encephalitis

CHAPTER I

LITERATURE REVIEW

I. Classsification

The term "arthropod-borne viruses" refers to viruses that are propagated in nature by hematophagous arthropods in an appropriate vertebrate host (W.H.O. Scientific Group, 1967). Over 400 viruses comprise this classification scheme (Calisher, 1980a). The arboviruses make up the preponderance of the family Togaviridae (Casals, 1957). Non-arbovirus members include rubella and equine arteritis virus. The togaviruses represent a group of small, enveloped, RNA viruses, that range in size between 40-70nm and are equipped with a symmetrical, icosahedral nucleocapsid (Wildy, 1971; Fenner, 1976). Within the family togaviridae two genera have been defined, the alphaviruses (formerly known as Group-A arboviruses) and flaviviruses (formerly Group-B). The genus alphavirus consists of 25 members, the majority of which replicate within a mosquito vector (Calisher, 1980a). Members of this group include Sindbis virus, Semliki Forest virus, as well as Eastern and Western equine encephalitis virus. Flaviviruses contain both tick-borne viral agents such as Langat and Louping ill virus, as well as mosquito transmitted viruses such as St. Louis encephalitis virus, Japanese encephalitis virus, and West Nile virus.

Several groups of investigators previously have reported on the serological overlap that exists among the alphaviruses (Howitt, 1935; Parks and Price, 1958; Karabatsos et al., 1963; Goldblum et al., 1972; Calisher et al., 1981). Dalrymple and his associates (1973) discovered that shared group antigenic determinants are located on the nucleocapsid, whereas envelope glycoproteins are virus specific and also function as subgroup associated

markers. Karabatsos detected significant antigenic variability within various strains of Western equine encephalitis (WEE) virus (Karabatsos et al., 1963), and also noted the existence of three distinct viral complexes within the alphavirus genus (Karabatsos, 1975). The concept of virus complexes, first described by Casals in 1957, represents a cluster of viruses within a serologically defined group, where certain members tend to be more closely related to each other than other members in the genus. WEE virus constitutes a complex along with Highlands J virus, Sindbis virus, Aura virus, Whataroa virus, Y62-33 virus, and Fort Morgan virus (Calisher et al., 1980b). Highlands J virus and Fort Morgan virus are closely related, while WEE virus is most similar to Sindbis virus. It is interesting to note that the latter two species are frequently the cause of active clinical infections, while the former species are rarely pathogenic. Recent advances in molecular techniques, such as oligonucleotide mapping and RNA fingerprint analyses, have allowed further differentiation of antigenic subtypes (Calisher et al., 1981).

II. Historical Perspective

For over 40 years Western equine encephalitis virus has been known to cause considerable morbidity and mortality in both equines and humans in North America. This virus was initially isolated by Meyer et al. in 1931 following a large epizootic in the San Joaquin Valley, California. Meyer and his co-workers were able to transmit the virus in laboratory animals by intracerebral and intranasal inoculation, although transmission to humans was not recognized at that time. A year later, Meyer (1932) realized the significance of this disease when he observed a similar encephalitic disorder in three men exposed to

infected horses isolated from an endemic focus. In 1933, two similar outbreaks of encephalitis were reported. In St. Louis County, Missouri, over 1,000 cases of human encephalitis became evident (Barr, 1934)while an equally large epizootic was recognized along the east coast (Hurst, 1934). The etiological agents were isolated and found to be serologically distinct from the western strain (Ten Broeck and Merrill, 1933; Cox and Fite, 1934). Based on their geographical distribution, these viral agents became known as Eastern equine encephalitis (EEE) virus and St. Louis encephalitis (SLE) virus. By 1938, Eklund and Blumstein were able to report six more cases of WEE by demonstrating the presence of neutralizing antibodies to WEE virus in these patients. The isolation of WEE virus from a human source was originally achieved by Howitt (1938). Shortly thereafter, Fothergill et al. (1938) and Webster and Wright (1938) were able to isolate the eastern strain from a human case. These results provided definitive proof establishing equine viruses as potential human pathogens. Subsequently, Schoening et al. (1938) suggested the need for further investigation to determine the natural mode of transmission of these viral agents.

Meyer (1932) noted a cyclical pattern of recurrence of the disease that was primarily limited to the warmer periods of the year. This led him to believe that biting insects could play a role in the dissemination of WEE virus. Kelser (1938) demonstrated that several species of Aëdes mosquitoes had the capacity to transmit either Eastern or Western virus in laboratory animals. Afterwards, Buss and Howitt (1941) reported cases of WEE occurring in rural areas associated with dense mosquito populations where irrigation ditches provided these insects with a natural habitat. Several species of mosquito are capable of carrying WEE virus, but Culex tarsalis has become recognized as

the primary arthropod vector in western North America (Hammon et al., 1941). The majority of mosquitoes other than Cx. tarsalis with the ability to transmit WEE virus function essentially as secondary conduits for this viral agent (Hess and Holden, 1958). There are however, endemic foci where other mosquito species may serve as the predominant vector.

Further research led to evidence implicating a number of small animals as potential hosts (Meyer, 1932; Olitsky et al., 1934; Cox et al., 1941; Burton et al., 1966b; Taylor, 1967; Hardy et al., 1974; Leung et al., 1975; Artsob and Spence, 1979). These animals typically develop high titred viremias, and hence were thought to represent "reservoir" hosts of WEE virus (Hammon, 1945). Eventually, Hammon et al. (1951) and Reeves et al. (1958) were able to provide sufficient data demonstrating wild birds as the major source of infection of WEE virus. Man and horses are thought to be accidental or dead-end hosts.

III. Natural Cycle and Distribution of WEE Virus

The ecological cycle of WEE virus necessitates that the virus must pass through a replication phase in an arthropod vector. This phase is termed the extrinsic incubation period. Infection of the vector usually occurs from ingestion of a blood meal from an infected host. However, several other mechanisms have been documented (Grimstad, 1983). The initial concentration of the viral pathogen is crucial if infection is to take place (Barnett, 1956; Thomas, 1963). Once infected, the mosquito has a limited period of infectivity during which it is capable of transmitting the viral agent (Kramer et al., 1981). Infection with WEE virus also demonstrates a unique seasonal pattern due to the restricted breeding and feeding season of its vector population. The

greatest number of infections are seen in July and August (Buss and Howitt, 1941). When the arthropod vector exhibits a viremic state, then the pathogen may be passed on to an unsuspecting host. Most infections generally arise in the western United States with extensions north into the Canadian prairie provinces (Fulton, 1938; Hess and Holden, 1958; Dillenberg, 1965; Kettyls, et al., 1972; Monath, 1979). Occasional focal outbreaks of WEE have also been reported in the eastern United States (Hayes and Wallis, 1977).

One of the puzzles that remains to be solved in this complex ecological sequence, is the process which allows the virus to escape lethal consequences during harsh environmental conditions. Several proposals have been put forward to account for this phenomena (Hammon, 1948; Sabin, 1958). Reeves et al. (1958) suggested that the reactivation of chronic latent infection in wild birds following a quiescent winter period may initiate the transmission cycle. A similar theory advocating a cyclical viremia, as seen in garter snakes, has also been proposed (Burton, 1966a). However, recent evidence indicates that transovarian passage of the virus in cimicid bugs may ultimately serve as the overwintering mechanism (Calisher et al.,, 1980).

IV. Morphology and Physical Characteristics

Much of the data dealing with the morphological aspects of alphaviruses has been garnered from studies of Sindbis and Semliki Forest virus. Studies pertaining to WEE virus have described it as a small virion, 40-55nm in size, enclosed within a lipoprotein envelope (Morgan et al., 1961). A single stranded RNA genome (mol. wt. $4x10^6$ daltons) is housed within a dense, central core particle. Irregular projections are associated with the surface of the virion

(Simpson and Hauser, 1968ab). Trent and Grant (1980) examined the structural proteins of WEE virus and determined the molecular weight of three major protein fragments. The E1 (envelope) glycoprotein has a molecular weight of 55,000 daltons, E2 47,000 and 33,000 for the capsid (C) protein. A small minor capsid protein is also involved in the structural make up of WEE virus (Ishida and Simuzu, 1981). The E1 glycoprotein can elicit hemagglutination-inhibition (HI) antibodies while the E2 fragment has been shown to induce virus specific neutralization (NT) antibodies (Dalrymple et al., 1973). The capsid proteins, which form the core particles, contain group-specific antigenic markers, and therefore induce the production of cross-reactive antibodies. Comparative amino acid sequence analysis between the proteins of WEE, Sindbis, and Semliki Forest viruses have disclosed a considerable degree of sequence homology (Bell et al., 1983). Such data supports the theory that many alphaviruses were derived from a common ancestor (Howitt, 1935). Schlesinger (1971) also proposed that many togaviruses have evolved from a common evolutionary link, but that due to intense selective pressures imposed by a replication cycle in both arthropods and vertebrates, these viruses have diverged from a group parent.

Proteolytic digestion of Group-A arboviruses has illustrated that WEE virus can be inactivated with trypsin treatment (Gorman and Goss, 1972). Loss of infectivity may be due to degradation of surface proteins responsible for the attachment of the virus to cell surface receptors (Kaariainen and Soderlund, 1978). A similar explanation has been put forth to account for the loss of hemagglutination activity following enzyme digestion (Horzinek, 1973).

A lipid bilayer, in the form of a "unit membrane", surrounds the virion (Morgan et al., 1961; Acheson and Tamm, 1967; Grimley and Friedman, 1970a). Since the lipid composition of the viral envelope is host cell dependent, it is not unusual to find a large degree of variability in its biochemical structure. The envelope consists primarily of neutral lipid most of which is unesterified cholesterol. Secondary lipids include phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, and phosphatidylserine (Pfefferkorn and Shapiro, 1974). All togaviruses have a lipid envelope, and consequently may undergo inactivation by organic solvents and detergents (Ventura and Scherer, 1970). Detergent degradation has been used to isolate and characterize various surface glycoproteins (Dalrymple et al., 1973; Trent and Grant, 1980). Alphaviruses may be inactivated by immersion into an acidic medium, but reveal increased stability in alkaline conditions. Exposure to high temperatures (56–60°C) will also inactivate a number of arboviruses. Greater stability may be achieved by placing the viruses in a protein solution at 4°C or freezing at -70°C for long term storage (Horzinek, 1973).

The development of WEE virus in a host cell is controlled by a unique set of nucleic acids which direct the replication sequence. In 1968, Sreevalsan and his co-workers reported the isolation of three specific forms of viral RNA. The three types of RNA, distinquished by their sedimentation coefficients in sucrose density gradients, consist of 40s, 26s, and 15s fragments. Biochemical analysis has revealed that the 26s form is a partial replicate analog of the 40s genome of WEE virus (Ou et al., 1983). The 40s form accounts for 99% of the total infectivity associated with the virion. The 26s RNA is thought to act as a mRNA that is responsible for the production of structural proteins (Garoff et al., 1982). By using temperature-sensitive mutants, the function of specific

viral genes has been elucidated (Hahimoto et al., 1977; Hashimoto and Simizu, 1978). Probes in the replication cycle of arboviruses have shown that a 140s structure exists in the cytoplasm of infected cells (Sreevalsan and Allen, 1968). The 140s structure is a precursor particle consisting of RNA and protein. The RNA portion sediments at both 40s and 26s, while the protein component is composed of nucleocapsid material. The nucleocapsid core particle eventually becomes coated with a lipid envelope derived by budding through the plasma membrane (Morgan et al., 1961; Acheson and Tamm, 1967; Scott and Burrage, 1984). Rocio virus, a flavivirus, obviates the need to bud through a membrane to aquire an envelope, as the virus is completely assembled within the cell (Tanaka et al., 1983). McGee-Russell and Gosztonyi (1967) provided supporting evidence that complete virus particles may be assembled within the cisternae of intracellular membranes and then released from the host cell.

V. Replication

The replication cycle of togaviruses has received a great deal of attention over the past few years since they have a small genome that can be easily characterized and are easily grown in a wide range of cell types including chick embryo fibroblasts and vero cells (Nir et al., 1957; Singh, 1972; Murphy, 1975; Mussgay et al., 1975).

Dulbecco and Vogt (1954), in one of the initial investigations of growth kinetics of alphaviruses, found that WEE virus replicates at a rapid rate with the first progeny becoming evident 1-2 hours post-infection. The virus passes through an exponential growth phase (Rubin et al., 1955), and after some 9-10 hours the first cytopathic changes become evident. These changes are

manifested by the formation of vacuoles and by shrinkage of the cytoplasm (Acheson and Tamm, 1967). At this stage virus production is at a plateau, and thereafter begins to decrease. In arthropod cells the picture is not so clear, as cytopathic changes are less frequent and infections commonly proceed to chronicity (Whitfield et al., 1971; Pfefferkorn and Shapiro, 1974). Chronic WEE virus infection of cell cultures can be achieved by prior exposure to inactive virus (Lockart, 1960). So called "infection barriers", which play a role in limiting the intensity of the infection, may contribute to the increased frequency of chronic infections in mosquito cell cultures (Kramer et al., 1981).

Initially, rapid adsorbtion of WEE virus takes place allowing the virion to enter the host cell (Dulbecco and Vogt, 1954). The virus is able to bind to cell receptors through the action of surface glycoprotein units (Garoff et al., 1982). Once inside the cell the virus releases its genomic RNA (42s fragment). This RNA serves as a mRNA to code for the production of an RNA polymerase (Ou et al., 1983). The genomic 42s RNA, termed the plus (+) strand, acts as a template for the transcription of a complimentary strand of RNA designated the minus (-) strand. The newly formed minus strand will in turn function as a template for the production of 42s genomic RNA and 26s RNA. The latter form provides the genetic information for the synthesis of viral structural proteins E1 and E2. The combination of these and other structural proteins results in the formation of a "spike glycoprotein complex". Translation of the structural proteins occurs on polysomes bordering the rough endoplasmic reticulum (RER), whereas the free ribosomes appear to be the site of translation of the capsid proteins (Kaariainen and Soderlund, 1978). The E1 and an E2 precursor protein, p62, are taken up into the RER where glycosylation takes place. The spike glycoproteins are assembled and transported via the golgi apparatus to the

plasma membrane. It is not clearly understood how the nucleocapsid particles are able to identify those regions of the membrane where the spike glycoproteins are located. Maturation by budding through the membrane allows the naked nucleocapsid to become enveloped by a lipid bilayer.

VI. Clinical Presentation

i) Symptoms in Equines:

The first sign of an outbreak of WEE is usually detected in sentinel chicken flocks or in the local equine population (McLintock, 1976). Since man may serve as a host for the pathogen, then early detection of the initial symptoms of this disease in horses is vital to the prevention of massive spread of the virus to the human population (Ecklund and Blumstein, 1938).

Initial reports in the literature described the incubation period of WEE in man as ranging between 5 and 21 days (McIntosh, 1938; Helwig, 1940; Shonefield and Townsend, 1953). The first recognizable symptom in horses is an elevation in temperature (Mitchell et al., 1938). Antibodies to WEE virus also become evident at this stage (Byrne et al., 1964). Furthermore, muscle tremors and rigidity of the cervical muscles may become obvious at this stage. As well, an impairment in balance and hypersensitivity to sound and touch have been documented (Sponseller et al., 1966). During the next phase, the animal becomes lethargic with a loss of coordination and develops a charactersitic "sleepy" appearance (McIntosh, 1938; Morgante et al., 1968). Both bowel and bladder functions may be lost. A sudden marked drop in temperature may be demonstrated, but the most obvious symptom is a progressive exhaustion leading to death.

ii) Symptoms in Man:

Patients suffering from WEE may display a number of signs and symptoms of varying intensity. Originally, it was believed that this heterogeneous group of symptoms were useful as diagnostic criteria for this particular disorder (Adamson and Dubo, 1942). Unfortunately, many of these unique symptoms may be shared by several of the viral encephalitides and other related disorders (Lennette and Longshore, 1951).

All age groups are susceptible to WEE virus infection (Hammon, 1941; Davison, 1943; McGugan, 1943). Infants, especially those six months of age or younger, demonstrate a higher incidence and develop a more fulminant infection (Platou, 1940; Herzon et al., 1957; Finley et al., 1967). By contrast, St. Louis encephalitis demonstrates a proclivity for older individuals (Barr, 1934; Jones, 1934). Sequelae are also noted more frequently in children following recovery from WEE (Bruyn and Lennette, 1953). Overall, WEE has a more favourable prognosis with a mortality rate of 5 to 15%, SLE is in the range of 10 to 30% and EEE approaches figures of 60-75% (Shinner, 1963; Adams, 1976; Leech et al., 1981). However, WEE virus is associated with high mild infection rates with the ratio of sub-clinical to clinical infections nearing levels of 1000:1 in the adult population (Johnson, 1982). Males are more likely to be infected than females, but this has largely been attributed to occupational and environmental factors rather than a genetic predisposition (Leake, 1944).

Folowing infection, the onset of clinical symptoms is rapid with a severe headache being the early hallmark feature (Weil and Breslich, 1942). Closely associated with this is a febrile period with "flu-like" symptoms such as myalagia, arthralgia, chills, nausea, and vomiting which can last from seven to

ten days. Two to three days after the initial symptoms appear neurological signs become apparent. Central nervous system involvement may be manifest by such signs as drowsiness, apathy, imbalance, stupor, and irritability. A stiff neck is also a frequent clinical observation. Speech difficulties may result due to the development of coarse tremors of the tongue. Patients frequently become delerious or disoriented, exhibiting various states of mental confusion. Convulsions are rare in adults, but quite common in young children (Medovy, 1943; Baker et al., 1958). Stiffness of the back is demonstrated on occasion, but muscle weakness and abnormal reflexes are the most constant physical findings. Transient paralysis is seen infrequently in adults. Incontinence of bowel and bladder occurs in most cases. Marked edema, bloating of the face, and visual disturbances such as photophobia are distinctive symptoms (Hammon, 1941; Adamson and Dubo, 1942). All of these symptoms usually go on to resolution once the temperature begins to return to normal. The acute phase of WEE lasts approximatly one week, with the average length of illness being three weeks (Gareau, 1941).

Patients infected by WEE virus, may succumb within 24 hours after initial onset of symptoms (Rozdilsky et al., 1968). Fortunately, the majority of cases recover with few residual effects (Finley and Hollister, 1951). Hammon (1945) has indicated that in the elderly renal and or pulmonary complications are common causes of death. In infants, fulminant infections can lead to death within two to three days. Muscular twitching, irritability, and increased tension or bulging of the fontanel are symptoms restricted to children suffering from WEE (Lennette and Longshore, 1951). Hypereflexia, opisthonos, spasticity, and paralysis are seen with greater frequency in children, thereby making the differentiation between poliomyelitis and WEE particularly difficult.

Analysis of the cerebrospinal fluid (CSF) can be helpful in making the diagnosis of WEE. A slight increase in pressure and protein may be detected in the CSF. The white blood cell (WBC) count may range from 100 to 300 cells per cubic millimeter. The preponderance of these cells are mononuclear being either lymphocytes or macrophages, though polymorphonuclear cells may be present. Early in the course of the disease polymorphs will predominate, but eventually the mononuclear cells become dominant (Hammon, 1941). Cohen et al. (1953) observed a similar trend except in their study the PMN cells were prominent in younger age groups, whereas older individuals demonstrated a lymphocytic response.

VII. Sequelae

As was pointed out by Noran and Baker (1943), the significance of a disease entity should not be judged solely on its lethal potential. Even though WEE has a relatively low mortality rate, studies have shown that the incidence of severe residual effects can be quite high (Palmer and Finley, 1956). This is particularly true for children less than one year of age where the frequency of sequelae may be well over 20% (Finley et al., 1967). Herzon and his associates (1957) have provide an excellent summary on the likelihood of developing long term complications following an acute infection by one of the major arthropod borne encephalitides. From this study, it is apparent that WEE virus plays a significant role in contributing to that fraction of the population suffering from neurological disorders attributed to viral infection. Since the frequency of sub-clinical WEE infections is quite high, it is important to establish those signs and symptoms that constitute sequelae in order to make the proper

diagnosis. The presence of many of these sequelae should not be considered unique to WEE, but rather to arboviruses in general (Brown et al., 1958).

In infants less than one month of age, over 50% of those infected have been shown to suffer some form of brain damage (Earnest et al., 1971). On the other hand, only 5% of the adult population suffering from WEE have definite signs of neurological impairment. Initially, Davis (1940) reported signs of deafness, blindness, mental deterioration, and spasticity as being indicators of a previous infection with WEE virus. Recurrent convulsive attacks are also indicative of a former infection (Noran and Baker, 1943; Baker, et al., 1958). Other forms of residua include difficulties in speech, rigidity, weight loss, and psychological dysfunction (Mulder et al., 1951). Finley et al. (1951) described patients with continuous headaches, tremors, and abnormal reflexes. Motor neuron disturbances are suggested by marked pyramidal and extrapyramidal symptoms along with partial paralysis and muscle weakness. Evidence of parkinsonism has also been found in a few cases, generally occurring as a latent symptom (Mulder et al., 1951; Palmer and Finley, 1956).

Finley and his co-workers (1967) were able to classify the residual effects as two types: 1) cognitive and 2) behavioural. The type and severity of the sequelae varied according to the length of the latency period subsequent to the acute attack. Their study indicated that when sequelae occurred a short time after the inciting infection, the residua commenced as behavioural disorders and gradually took on cognitive features. Cognitive impairment was the principle late sequela. The incidence and severity of these residua were also correlated with the presence of convulsive attacks during the acute phase (Finley et al., 1955). The authors concluded that an acute infection with WEE virus in a young child will result in aberrations in cerebral development leading

to intellectual and psychological deficiencies. Bruyn and Lennette (1953) have also cited WEE virus as the possible causative agent for many obscure neurological disorders in children. A similar argument was put forward by Fulton and Burton (1953), who claimed that much of the mental instability seen in Canadian mental institutions could be the result of asymptomatic infections with WEE virus. These speculations remain unproved.

VIII. Pathogenesis of WEE Virus

The pathogenesis of arboviral agents invading the central nervous system (CNS) has been intensively studied (Hurst, 1934; McCordock et al., 1934; Smadel and Moore, 1934; Quong, 1942; Zimmerman, 1946; Kissling and Rubin, 1951; Albrecht, 1957b; Jungherr et al., 1958; Shinner, 1963; Miyake, 1964; Yasuzumi et al., 1964; Yusuzumi and Tsubo, 1965; Craighead et al., 1966; Seamer et al., 1967; Murphy et al., 1968; Liu et al., 1970; Weiner and Cole, 1970; Reyes et al., 1981; Shankar et al., 1983). Unfortunately, there are still many gaps in our understanding of the pathological process. Several factors might contribute to CNS invasion by a virus. These factors include the virulence of the particular viral strain, the host defense, and the route of entry into the brain (Albrecht, 1968). The bulk of the research has attempted to elucidate the various pathways available for viral entry into the brain (Sabin, 1938; Sabin and Olitsky, 1938; Friedmann, 1943; Johnson, 1963, 1965; Huang and Wong, 1963; Nir et al., 1965; Ojeda, 1980; Grimley, 1983; Monath et al., 1983; Kristensson et al., 1984a).

i) Pathways of Virus Spread Into the Brain:

Johnson and Mims (1968) have provided an excellent review elaborating the possible routes of viral entry into the CNS. There appear to be three major pathways, the hematogeneous route, the neural route, and the olfactory route. The route taken following a natural infection will depend upon: 1) the route of inoculation, 2) the original site of viral replication, 3) the infecting viral dose, and 4) the host defense mechanisms (Grimley, 1983).

A neural route was readily perceived due to the anatomical connections between the peripheral and central nervous systems. Studies carried out on rabies virus have become the hallmark examples of virus transmission via nerve tracts (Murphy et al., 1973a). Using immunofluorescent techniques, other viruses such as herpes simplex virus have also been identified along neural pathways (Johnson, 1963). This virus appears to travel down the nerve by infecting endoneural cells such as Schwann cells. Eventually, it was found that axonal transmission and spread via neural lymphatics could also take place (Janssen et al., 1984).

Migration of virions from the nasal region to the olfactory bulbs was established long ago (Brodie, 1934; Sabin, 1938). However, the exact mode of transmission along the olfactory route continues to be the centre of debate (Monath, 1983). Originally, Sabin and Olitsky (1938a) suggested that this was the sole pathway into the CNS. This theory was boistered by evidence from Hurst (1936), who pointed out that virus need not be inoculated directly into the nasal region, but could reach the olfactory gland by the peripheral circulation. Following intranasal inoculation sequential analysis of brain tissue taken at varying stages following infection indicates that the olfactory bulbs are in fact the original site of viral replication within the CNS (Webster and

Chow, 1936; Albrecht, 1967b; Monath et al., 1983). When travelling along the olfactory system, there are two pathways which can be taken. Arboviruses may infect the nasal mucosa, extend into the sub-arachnoid cuffs and cause a diffuse infection of the meninges. The second and more likely route, involves the invasion of neural cells associated with the olfactory fibres which enter directly into the brain. Research has shown that Herpes simplex virus is capable of travelling along the olfactory nerve into the brain (Ojeda, 1980). Nasal secretions have also been implicated in the transmission of arboviruses among man (Froeschle, 1964; Monath et al., 1983). Mindful of the fact that most arthropod-borne viruses are deposited into the human circulation by mosquito vectors, the olfactory route likely plays only a minor role in the dissemination of viruses into the cerebral tissue (Johnson, 1963).

The major pathway of virus spread into the CNS is thought at present to occur via the bloodstream (Johnson, 1982). Hematogeneous spread requires a sufficient level and duration of viremia to overcome the host defense systems. Extensive viral replication at an extraneural site must take place in order to maintain the viremic state. Nir et al. (1957) illustrated the vascular endothelium as a prime area of intense viral growth. Thus, viruses proliferating in the endothelial lining of the cerebral blood vessels could easily grow through the vessel wall into the CNS (Johnson, 1965; Cole et al., 1970). Nevertheless, there are numerous instances where virus proliferation in vessel walls has not been implicated (Bhamrapravity et al., 1964; Mims, 1964). Consequently, many secondary sites of extraneural infection have been investigated including muscle tissue, heart, spleen, pancreas, intestines, and brown fat (Murphy et al., 1973b; Harrison et al., 1980, 1982). Among this group, virus growth in muscle tissue has generated the most interest (Grimley

and Friedman, 1970b). Once the virus gains entry into the host, multiplication occurs primarily in muscle tissue at the site of entry. Virus progeny will invade the lymphatic system and subsequently enter the systemic circulation (Janssen et al., 1984). If the infecting inoculum is of a sufficiently high dose, viral replication may reach adequate levels to maintain a viremia, thereby allowing infection of the cerebrovascular endothelium. In a previous study, Wong (1963) lent support to this theory when he was able to demonstrate that a peripheral growth phase was required before infection of the CNS could take place. If the pathogen is virulent an active encephalitic process will ensue (Monath and Trent, 1981).

Passive transmission of virus particles across blood vessels may also explain seeding of the grey and white matter (Albrecht, 1960). This concept has been attributed to an alteration in the electrochemical potential at the site of the barrier which results in an increase in membrane permeability (Friedmann, 1943).

The blood-cerebrospinal fluid route provides an alternate pathway into the brain. This route has come under close scrutiny by researchers because of the porous endothelial lining of the capillaries within the choroid plexus. If virus particles can escape from the circulation through the vascular plexus into the CSF they may enter the ependymal cells which line the ventricles of the brain thence reach brain parenchymal cells. Several reports have indicated that ependymal cells and epithelial cells of the choroid plexus are intimately involved in the infection process (Hamashima et al., 1959; Liu et al., 1970; Shimokata et al., 1976; Kristensson, et al., 1983; Janssen et al., 1984). This evidence is strongly suggestive that the movement of viruses occurs from the bloodstream to the brain by way of the blood-CSF route.

Two other modes of entry which have not received a great deal of consideration are the enteric and transplacental pathways. Transmission of arboviruses across the intestinal mucosa seems to be an unlikely event. Since arboviruses are composed of a lipid envelope, the probability that they could withstand the harmful effects of digestive enzymes and bile are very remote (Ventura and Scherer, 1970). On the other hand, poliovirus which does not have a lipid envelope, can penetrate through the intestinal mucosa resisting the damaging effects of enzymes, acids, and bile by virtue of a tightly knit nucleocapsid shell.

Evidence demonstrating transplacental transmission suggests that this is a viable route of inoculation (Cohen et al., 1953; Shonefield and Townsend, 1953; Copps and Giddings, 1959). Since fetuses infected with WEE virus may suffer severe malformations (Moreland et al., 1970ab), this route should not be dismissed. The matter requires further study.

ii) Host Response to Neurotropic Viruses:

Once viruses reach the peripheral circulation they become exposed to a full array of human defense mechanisms. Macrophages effectively clear the virion from the bloodstream reducing the viremia and lowering the probability of secondary infection of the CNS. McFarland et al. (1972) demonstrated that the mononuclear cells which respond to a Sindbis induced encephalitis in mouse brain are virus specific.

Interferon production has also been postulated as a means to control neurotropic viral infections, but the evidence has been inconclusive (Cole and Wiseman, 1969; Shimokata et al., 1976)

Neutralizing antibodies are also seen as effective against viral infections of the CNS. Howitt (1932) and Zichis and Shaughnessy (1940) demonstrated the prophylactic potential of immune sera in the treatment of WEE. They noted a significant reduction in the intensity of infection following the administration of immune sera during the acute stage. Virions are also cleared at a faster rate when pre-mixed with immune sera (Mims, 1964). Jahrling et al. (1983) suggested that opsonins were the key elements in inducing rapid phagocytosis of virus particles from the circulation. Schlesinger (1949) inferred that local antibody production was important in preventing widespread viral invasion. Morgan et al. (1942) had previously reported that only sufficiently elevated levels of antibody would confer immunity in a given host. The majority of host immune mechanisms are helpful in staving off neurotropic viruses but as pointed out by Camenga and Nathanson (1975), some immune responses may be more deleterious than the actual infectious process.

Resistance to viral attack may also be a function of genetic determinants. Sabin (1952) was able to show that certain inbred mice were resistant to flavivirus infection due to an autosomal dominant allele. Other factors which are known to contribute to the overall pathology of neuroinvasive viral infections include the age of the host, the infecting viral dose, and the virulent character of the virus (Craighead et al., 1966). The relationship between age and the development of disease has been studied most extensively (Olitsky et al., 1934; Sabin, 1938; Gleiser et al., 1962; Grossberg and Scherer, 1966; Seamer and Randles, 1967).

Generally speaking, immature or newborn animals tend to be much more susceptible to arboviral attack than older individuals of the same species. This phenomenon was originally attributed to an increased resistance of the mature

host due to the development of localized protection barriers (Sabin and Olitsky, 1938a) thought to be situated in blood vessels, in the olfactory region, and at myoneural junctions. However, sophisticated ultrastructural techniques have failed to reveal their presence.

Immaturity of the immune system in a newborn may also be responsible for the increased susceptibility to disease. Johnson (1964) reasoned that in older animals the macrophages were more functionally mature, and therefore could proceed more efficiently in clearing viral particles from the bloodstream. A heightened antibody response may also account for the immune status in these hosts, but the evidence for this has been conflicting (Morgan et al., 1941). It is also beleived that high viral multiplication rates, associated with increased virulence, may overwhelm immature defense systems leading to high titred viremias which are more likely to produce CNS involvement (Eldadah et al., 1967). Virulence, may therefore be a reflection not only of increased replication rates but also the presence of efficient cell receptors (Cole et al., 1970; Janssen et al., 1984). The extent of the inflammatory lesions seen in a histopathological picture will vary according to the intensity of the encephalitis. In fulminant infections the lesions will be minimal.

Dosage is another variable that can influence the course of viral infections. Grossberg and Scherer (1966) postulated that large viral inocula could saturate the reticuloendothelial system (RES). This would allow inumerable viral particles to exist in the circulation so as to negate any appreciable effects by actively phagocytic macrophages. Secondary neural invasion will increase in probability following a substantial initial infection (Wiener et al., 1970).

iii) Viral Passage Within the CNS:

Once an invading viral agent has reached the central nervous system, three avenues of further spread are available. Firstly, virus dispersion may take place through the extracellular spaces of the brain parenchyma. Electron microscopic studies of the neuropil have shown that these spaces are approximately 10-15 nm in size (Wolinsky and Johnson, 1980). Arboviruses are in the order of 40-70 nm in size. Therefore, arboviruses appear to be too large to pass through these spaces. However, Group-A and Group-B viruses have been detected within the intercellular gaps (Blinzinger and Muller, 1971; Bastian et al., 1975). Fixation artifact was believed to be responsible for the discrepancy in size between the virus and the intercellular spaces. Therefore, according to these studies the intercellular spaces may function as pathways for the dispersal of virions within the brain.

A readily apparent method of viral propagation within the CNS involves the transfer of viruses in a cell to cell manner. Grimley and Friedman (1970a) explained widespread localization of virions in brain tissue as occurring via axoplasmic transport systems. Focal areas of infection can be envisaged following this route. Widespread areas of infection can also occur by this system provided that susceptible neural elements are linked by long cytoplasmic processes (Johnson and Mims, 1968). Immunofluorescent studies of West Nile virus have also suggested axonal transmission as a means of viral propagation (Eldadah and Nathanson, 1967). Furthermore, the presence of viral antigen in peripheral nerves late in the infection strongly suggests centrifugal spread.

The final route for viral dissemination in the brain resides with the role of glial cells. These cells can function as migrating foci of infection, carrying the pathogen to distant sites within the nervous tissue (Mims, 1960; Blinzinger and Muller, 1971). This concept has been received with much skepticism due to the minimal evidence supporting it.

iv) Susceptible Cells Within the CNS:

Not all cells within the central nervous system are equally susceptible to viral infection. For instance, poliovirus attacks motor neurons, whereas herpes simplex virus is capable of infecting a wide variety of cell types (Johnson and Mims, 1968; Wolinsky and Johnson, 1980). Arthropod-borne viruses infect a limited population of neurons within the CNS which results in a characteristic clinical picture (Nathanson et al., 1966; Finley et al., 1967). Areas demonstrating particular susceptibility to viral infection include the substantia nigra, the vestibular nuclei, the cerebellar cortex and the anterior horn cells. Reyes et al., (1981) also believe that specific nuclear groups are more prone to infection with viral agents and that these groups may be useful as "indicator centres" serving to distinquish recent viral encephalitides. Other nuclear groups which have shown heightened sensitivity to viral invasion include the hippocampus, cerebral cortex, hypothalmus and the dorsal nuclei of the medulla (Kissling and Rubin, 1951). Other factors thought to contribute to the increased susceptibility of neural sub-populations include the degree of cellular differentiation, the mitotic rate of a given cell population, and their access to readily available portals of entry such as a local nerve fibre (Johnson, 1980).

IX. Pathological Features of WEE Virus Infection

i) Gross Pathology:

WEE virus may infect several visceral organs including the liver, spleen, kidneys, skeletal muscle, adrenals, and salivary glands (Howitt, 1932; Aquilar, 1970; Liu et al., 1970; Leung et al., 1978). However, the most serious pathological consequences occur when this virus behaves as a neurotropic agent attacking the central nervous system. Gross examination of brains from patients dying soon after an acute infection attributed to WEE virus reveals edematous cerebral tissue with congested blood vessels. The latter are most prominent on the surface of the cerebrum and the meninges. Petechiae are also common findings at autopsy and occasionally subarachnoid bleeding and a bloody CSF may be found (Baker and Noran, 1942).

Noran and Baker (1943) cited the presence of several cysts in the frontal and temporal regions of the brain of a child with chronic WEE. The cysts were separated by a thin wall of fibrous tissue. Baker et al. (1958) believed that these cysts were the result of a thickening of vascular walls and a secondary oligemia. These cysts have been an infrequent finding and in fact are likely to represent long term patterns of degeneration. In some instances, there are no pathological lesions in the nervous tissue (Anderson, 1984).

A possible secondary complication of WEE is the development of a mild pneumonitis (Rozdilsky et al., 1968). An inflammatory response may also be observed in other organs (Aquilar, 1970). The intensity of the reaction will vary with the site of inoculation (Nir et al., 1957; Liu et al., 1970), but the distribution of the viral agent in the tissue remains the same (Albrecht, 1968).

ii) Micro-anatomical Pathology:

WEE virus produces a typical histological picture following infection of the nervous system. The pathological features have been observed in numerous animal species including burros, horses, mice, squirrels, and man (Binn et al., 1966; Sponseller et al., 1966; Aquilar, 1970; Leung et al., 1975, 1978; Anderson, 1984). On occasion, experimentally infected infant mice have failed to reproduce the typical signs of encephalitis, whereas other animal species tend to develop the complete microanatomical derangements (Liu et al., 1970).

The classic histopathological signs of viral encephalitis include the localization of inflammatory cells around blood vessels (perivascular cuffing), along with scattered regions of focal neuronal necrosis (Adams, 1973). Astrocyte proliferation and nests of microglial cells are also typical features of the CNS during an encephalitic attack (Noran and Baker, 1942; Zlotnik, 1968). In WEE, perivascular cuffs or collars are diffusely located throughout the brain (Peers, 1942). These collars of cells consists mainly of lymphocytes and macrophages but polymorphonuclear cells (PMN) may also be present (Baker and Noran, 1942). The cells may invade the adventitia and lead to distention and congestion of the perivascular space (Quong, 1942). Large veins tend to accumulate lymphocytes, while the smaller vessels become encircled by macrophages (Baker et al., 1958). It also appears that polymorphonuclear cuffs are more prevalent in the grey matter, whereas macrophages and lymphs predominate in the white matter (Baker and Noran, 1942). When PMN cells infiltrate a blood vessel a microabscess may form in the surrounding tissue. Cyst formation and the development of microabscesses were thought to be characteristic features of WEE, but these lesions have also been described in other arboviral encephalitides (Zimmerman, 1946; Shankar et al., 1983).

Hyperplasia of the endothelial lining of cerebral vessels is commonly associated with acute viral encephalitis (Noran, 1944; Nir et al., 1957). The intimal lining may expand to such an extent that vascular occlusion results. Suppurative thrombi due to leukocytic infiltration have also been documented (Noran and Baker, 1945). In chronic cases of WEE, hyalinization and subsequent calcification of vessels has been known to occur (Noran and Baker, 1943). These authors have suggested that the cellular infiltrate, in accompaniment with endothelial proliferation, leads to vascular ischemia and predisposes the nervous tissue to necrotic damage. This data would support the theory that vascular occlusion is a primary response to viral replication followed by secondary nerve cell damage. Several authors have put forward an opposing point of view stating that perivascular cuffing is a secondary phenomena following initial neuronal destruction (Weil and Breslich, 1942; Zimmerman, 1946; Finley and Hollister, 1951; Shinner, 1963).

Many of the early degenerative changes seen in neurons occur in those populations intimately associated with blood vessels. Several investigators have offered this as proof that WEE virus is transmitted via a hematogeneous route (Larsell et al., 1934). The damaged neurons may be swollen or shrunken and have an eccentric or pyknotic nucleus (Weil and Breslich, 1942). Neurons localized in damaged areas have been shown to contain aggregated neurofibrils along their cell wall (Peers, 1942). Focal areas of necrosis may be inundated with lymphocytes, polymorphs, and microglial cells (Adams, 1976). Quong (1942) observed numerous microglial "rod cells" in necrotic centres while investigating an outbreak of WEE in Canada. These necrotic foci appeared as moth-eaten lesions usually associated with spongiform encephalitides. The vacuolar lesions may be up to 1mm in diameter and are situated only in the grey matter (Peers,

1942). Such lesions are much more prominent in EEE (King, 1941). Several scattered microglial nodules or nests containing intact neurons can be visualized in cases of WEE. Gliomesenchymal nodules are also a common finding in arboviral infections of the nervous system (McCordock et al., 1934; Wesselhoeft et al., 1938). These sites can exhibit mitotic figures and may vary in their density and location. The white matter contains fewer and more compact nodules which lie in close proximity to blood vessels. Finley and Hollister (1951) determined that PMN cells were associated primarily with young nests of microglia. The microglial cells phagocytose degenerating neurons by a process known as neuronophagia leaving a clear zone in the neural tissue.

Zlotnik (1968) noticed a marked degree of astrocytic hypertrophy and hyperplasia during the acute infection stage. The astrocytes became prominent prior to an inflammatory reaction. Foci of astrocytes can coexist with areas of sub-acute polymorph aggregation (Baker and Noran, 1942), but can undergo dissolution along with the other cellular elements in the local environment.

Two pathological signs which complete the histological picture of WEE are petechial hemorrhages and foci of demyelinization (Noran and Baker, 1945). Hemorrhages if present, can usually be detected on the surface of the brain during gross inspection. Demyelinization is restricted to a few regions in the brain may be seen with destruction of axis cylinders (Noran and Baker, 1943).

Early investigators believed that the presentation of the major features of WEE were in themselves pathognomonic (Noran and Baker, 1945). However, the study of other encephalitic disorders has demonstrated considerable overlapp in pathological features, thereby making the histopathological diagnosis of WEE extremely difficult (Lennette and Longshore, 1951).

X. Localization of Virus and Pathological Lesions in WEE

The pathological features of WEE by themselves may not be pathognomonic, but when they are associated with a distinct distribution pattern they may be utilized to differentiate this disorder from other forms of encephalitis.

Peers (1942) was the first to carry out an extensive study of the anatomical distribution of the lesions found in WEE. His investigation revealed a moderate number of lesions on the dorsal and anterior aspects of the cerebral cortex. This observation has been confirmed by numerous other studies (Quong, 1942; Rozdilsky et al., 1968). Olfactory bulbs may become infected depending upon the route of inoculation and the duration of the infection (Albrecht, 1957b). Various authors (Sponseller et al., 1966; Leech et al., 1981) have found that the occipito-parietal area is more severely affected than the temporal and frontal region of the cortex. These results are somewhat similar to the findings of Noran and Baker (1943), who reported marked nerve cell damage, mild astrocytosis, and intense vascular congestion in the tempo-parietal region of the brain with less extensive damage in the occipital region. Isolated perivascular cuffs and areas of partial demyelinization may also be seen in the white matter of the temporal lobes.

Sub-cortical damage may be quite prominent and is particularly extensive in the basal ganglia (Noran, 1944). Occasional glial nests may be present in the sub-cortical white matter of both the cerebrum and cerebellum (Finley and Hollister, 1951). In the basal ganglia, the anterior portion may be more severely involved, nonetheless the putamen, the caudate nucleus, and the grey stripes can also exhibit extensive histopathological lesions. Conversely, the

anterior perforated substance and the amygdaloid nuclei offer little evidence of an encephalitis. Thick-walled blood vessels with advanced stages of hyaline formation are commonly visualized in the basal ganglia, the cerebral white matter, and dentate nuclei of patients suffering with WEE (Leech et al., 1981). The globus pallidus and the periventricular grey matter may have few glial nodules and moderate numbers of necrotic nuclei. Generally speaking, neuronal involvement is less evident in the corpus callosum and fornix. If the infection is relatively severe, than a few lesions may become evident in the anterior corpus callosum (Noran and Baker, 1943).

Several investigations have demonstrated that the corpus striatum is the site in the brain most likely to show signs of encephalitis following infection with WEE virus (Liu et al., 1970; Anderson, 1984). Necrotic neurons are commonplace, especially in close proximity to blood vessels of the cortex in the white matter of the corpus striatum. Proliferation of astrocytes is a constant observation in this region of the brain. Evidence of viral replication has also been seen in the ependymal cells lining the ventricles and the endothelial cells of the choroid plexus. Overall, the thalamus has fewer lesions than the basal ganglia. Foci of microgliosis can be appreciated in the thalamic nuclei with a few rare foci in the sub-thalamic, maxillary, and lateral geniculate bodies.

The hippocampus shows extreme variability in its response to an attack by WEE virus. The degree of cerebral involvement in this region corresponds directly with the intensity of the the acute disorder. The pyramidal cells are the major site of viral replication. In fulminant cases, glial nodules may appear in the small cell layer of the dentate gyrus. Still, it is not uncommon to find that the hippocampal region of the brain is completely spared.

In the midbrain, the substantia nigra is the area that is chiefly affected. Small focal lesions may be seen in the grey matter and around the aqueduct. The red nuclei may also be involved. Children will present with a moderate number of lesions in the substantia nigra, whereas adults generally demonstrate a more widespread distribution.

In the pons, there are a few isolated necrotic nerve cells with damage being limited to the inferior olives, the dentate nuclei, and the nuclei of the tegmentum. In the base of the brain, a moderate number of foci in the intrafascicular grey matter are observed.

The cerebellar cortex may contain large glial nests surrounding a rare necrotic Purkinje cell. This results in the formation of a clear zone. Large nodules tend to be prominent in the dentate nuclei. Vertical streaks of microglial nodules have also been described in the molecular layer (Peers, 1942). In contrast, EEE displays few histopathological changes in the cerebellum (Kissling and Rubin, 1951; Dent, 1955).

Both sensory and motor neurons in the medulla are affected. A few large glial nodules may form in the grey matter and in the hilus of the inferior olives which in turn appear as swollen bodies with pyknotic nuclei. Small areas of glial hyperplasia are located in the pyramids and rentiform bodies. A few scattered nodules exist at the midpoint of the medulla in the grey and white matter.

The spinal cord may have severely damaged axis cylinders. There are focal and diffuse accumulations of PMN cells and lymphocytes with some cuffing of blood vessels. The lesions are sequestered primarily in the grey matter of the anterior and posterior horns. Sponseller et al. (1966) reported a moderate degree of neuronophagia, satellitosis, and aggregation of neutrophils.

The ganglion cells of ventral horns are only mildly affected. Demyelinization is patchy or not evident in most human cases of WEE. A few glial nodules can be seen in the reticular process and nearby within the base of the anterior horns.

Immunofluorescent investigations indicate that at the cellular level specific arboviral antigen fluorescence is limited primarily to the cytoplasm of infected neurons (Eldadah and Nathanson, 1967; Liu et al., 1970). Fluorescein tagged antibodies have further localized viral antigen in neuronal dendrites and axons. These authors have also reported sighting a few infected microglial cells. In a few instances, arboviral antigens appeared to be intranuclear (Hamashima et al., 1959; Yasuzumi and Tsubo, 1965; Cole et al. 1970; Harrison et al., 1980).

XI. Diagnosis of Arboviral Infections

Conventional methods utilized for the diagnosis of arboviral infections consist of isolation techniques and serological analysis. Today, several alternative procedures may be employed to determine the causative agent of an encephalitis. These techniques include immunofluorescence staining, electron microscopy, immunoelectron microscopy, immunoenzyme assay, and radioimmunoassay (Casals, 1979).

i) Standard Methods of Diagnosis:

The specific diagnosis of WEE and other arboviral encephalitides requires both the isolation of the virus pathogen from a clinical specimen taken during the acute phase of the disease, and the demonstration of a significant serological response. Unfortunately, recovery of an arbovirus is a rare event

due to a number of variables associated with the isolation procedure. In order to isolate the virus from a clinical specimen, that specimen must be collected at the appropriate time during the course of the disease and must be handled properly to maintain the viability of the pathogen. In arboviral infections, the symptoms begin to manifest themselves at the end of the viremic stage. As a result virus isolation from a blood specimen is almost never achieved. The specimens which are collected at appropriate intervals are frequently improperly handled. They may be exposed to a variety of temperatures and transported for long periods of time, thus reducing the chances of viral recovery. In a few instances, CSF and throat swabs may yield virus. However, brain tissue serves as the optimal source of the pathogen. Biopsy or autopsy material is homogenized in a suitable diluent and inoculated into a susceptible host species or cell culture. Intracerebral inoculation of suckling mice (1-3 days old) is the accepted method, although other animals such as hamsters and chick embryos have been utilized. Commonly used cell types for viral isolation include chick embryo fibroblasts, vero cells and mosquito cell cultures (Monath and Trent, 1981). Infected host animals or cell cultures are closely monitored. Sick or moribund animals are sacrificed and suspect infected organs are utilized to reinoculate the same host species until an elevated viral titre is achieved. Cell cultures will demonstrate cytopathic effect (CPE) or infectivity plaques. Fluorescent antibody assays or immunoperoxidase methods may be used to detect replicating viral agents in tissue culture. Following serial passage in a susceptible host which consistently demonstrated recognized signs of illness, the tissues and sera may be tested by hemagglutination or complement fixation tests to identify the serotype. To confirm the diagnosis neutralization tests will be performed. Rarely used methods of identification include size

determination by filtration through a porous membrane, or sensitivity to ether, deoxycholate, and acids.

Three major serological techniques are available for the identification of virus isolates. These are neutralization (NT), complement-fixation (CF), and hemagglutination-inhibition (HAI) tests. These assays can detect the presence of specific antibodies where demontration of a rising or falling antibody titre between an acute and convalescent serum sample will confirm the diagnosis.

The complement fixation test is an extremely useful diagnostic technique in arbovirology. The CF test, first applied to the diagnosis of neurotropic viruses by Casals and Palacios (1941), can detect antibodies which appear for only a brief period of time. Since CF antibodies do not begin to appear until two to three weeks after initial infection, this method cannot be used as a rapid diagnostic procedure. Nevertheless, one of the advantages of this test is that it allows the differentiation of closely related species within a serological group, that is it is type specific. Among the difficulties associated with this assay is the development of anticomplimentary activity in sera, misinterpretation of results due to immunological overlap, and the lack of sensitivity in determining previous infections.

The hemagglutination-inhibition test serves as a screening procedure in the identification of arbovirus infections. For the most part HAI antibodies are group specific, but in the case of alphaviruses these antibodies may on occasion be type specific (Monath and Trent, 1981). HAI immunoglobulins will peak early and then fall rapidly before levelling off. These antibodies can be detected for several years. Non-specific inhibitors of agglutinins can be found in normal sera. The inhibitors are mainly lipids and lipoproteins which can be

removed by adsorption to kaolin or by inactivation with protamine sulphate. The removal of these inhibitors can however alter the antibody titre.

The neutralization test is the most type specific serological assay of the three tests. This procedure is based on the ability of host antibodies to neutralize a standard suspension of virus present in a host animal or cell culture. Neutralizing antibodies appear early and are extremely long lasting. Similar to the HAI test, non-specific inactivating substances may be present in human sera, thereby requiring heat inactivation. The severest limitation of this procedure is the length of time required to obtain a result.

ii) Recent Advances in Arbovirus Diagnosis:

Immunofluorescent (IF) antibody techniques have gained an increasing amount of use in the detection of arboviral antigens (Bhamrapravata et al., 1966; Liu et al., 1970). Fluorescent antibody detection of arboviral antibodies has also been documented, yet remains an infrequently used procedure (Emmons et al., 1969). Emmons and Lennette (1966) were able to use this technique for the rapid diagnosis of Colorado tick fever (CTF) virus in blood smears. Immunofluorescent staining has also been used to detect the presence of SLE, EEE and Herpes simplex viruses in the brains of encephalitic humans and equines (Tomlinson et al., 1974; Reyes et al., 1981; Monath et al., 1981). These studies helped to establish the value of this technique as a rapid diagnostic method for determining the etiological agent responsible for an encephalitic disorder. IF assays may also be used to study the pathogenesis of viral infections, especially those affecting the nervous system (Kristensson et al., 1983; Kristensson et al., 1984b). The greatest advantage of this method, undoubtedly lies in its ability to provide results within a short period of time.

Disadvantages include the capital cost of the fluorescent microscope and the need for highly trained personnel capable of interpreting findings with confidence.

Electron microscopy (EM) and immune electron microscopy (IEM) have not played a significant role in the diagnosis of arboviral infections. For EM studies to be diagnostic the viral agent must present with a distinct morphological structure and be associated with the appropriate clinical syndrome. Many arboviruses share common structural features, and therefore cannot be positively identified by electron microscopy (Smith, 1978).

Immune electron microscopy has proven to be useful for the diagnosis of arbovirus infections, including WEE (Flauvell, et al., 1977). Even though this procedure is rapid and specific, it has yet to reach its full potential as a diagnostic method. Once again, the major drawback are the cost and the requirement for highly trained personnel to operate the microscope and interpret results.

Radioimmunoassays (RIA) have been utilized primarily for the detection of arboviral antibodies (Levitt et al., 1976; Jahrling et al., 1978). This has prompted many laboratories to establish these methods as their routine procedures for the detection of diagnostically significant antibodies. Results obtained by the RIA method are specific, accurate, and available within a few hours of testing. The difficulties associated with this assay include the cost of purchasing radioisotopes and a scintillation counter, the limited shelf life of the reagents, and the proper disposal of radioactive wastes.

Enzyme antibody methodologies in the form of immunoperoxidase (IP) and enzyme-linked-immunosorbent assays (ELISA), have become important tools in the diagnosis of viral diseases (Wicker and Avrameas, 1969; Kurstak, 1971;

Grandien and Olding-Stenkvist, 1984). These procedures depend upon enzyme antibody conjugates linking up with specific antigens to form coloured end products. Thus, a specific antigen can be demonstrated to exist at each site of focal colouration.

The immunoperoxidase assays consist of several closely related procedures all of which are based on an enzymatic reaction between horseradish peroxidase (HRP) and an appropriate substrate (Sternberger, 1979). The method to gain the most widespread acceptance is the peroxidase-antiperoxidase (PAP) procedure developed by Sternberger et al. (1970). The culmination of this procedure results in a complex conglomerate of antigen, antibody, and antibody-enzyme. Once such a structure has been assembled, visualization of a specific antigen may be accomplished through a relatively simple enzymatic reaction. The PAP method has been utilized most effectively in the area of diagnostic pathology for the identification of variety of cellular antigens (Elias, 1982; Falini and Taylor, 1983). Recently, viral antigens have also been detected with the PAP technique (Stephens et al., 1977; Tabuchi et al., 1978; Kohana et al., 1981).

Immunoperoxidase methods offer several distinct advantages over other diagnostic procedures. Many of the inherent advantages of the PAP technique can be attributed directly to the nature of the enzyme-antibody conjugates. Unlike fluorescein tagged antibody preparations, the conjugates prepared for immunoperoxidase staining can be easily coupled and are stable for long periods of time. This not only enhances the sensitivity of IP assays, but also reduces costs because of the increased shelf life of the reagents. As mentioned previously, the enzyme most often coupled to the immunoglobulin is horseradish peroxidase. Since this enzyme has a low molecular weight the overall size of

the conjugate is reduced, thereby enhancing penetration into the tissue and increasing the degree of cellular detail. This enzyme can be obtained cheaply from a number of sources and is able to withstand harsh histological procedures while retaining its functional activity (Sternberger, 1979).

The final product of the PAP reaction is a stable opaque granular deposit. Diffusion of the end-product from the site of reaction is limited, and therefore precise localization is achieved. Secondary or post-fixation of the specimen may be carried out with osmium tetroxide rendering the deposit electron dense. Consequently, visualization of the antigen at the ultrastructural level is possible with the use of the electron microscope. This is particularly useful in studies which are concerned with the events of viral replication (Ubertini et al., 1971; Shabo et al., 1972; Dubois-Dalcq and Barbosa, 1973). Even though IP methods can be adapted to electron microscopy their true value lies in their applicability to routine light microscopy. The cost of expensive ultraviolet and electron microscopes can be ommitted by using IP assays. Frozen tissue, paraffin, or plastic embedded tissue can all be used with IP techniques, whereas IF procedures usually require frozen sections. Tissue preparation is facilitated by the use of routine paraffin embedded tissue, and retrospective studies may be carried out using tissues prepared in this fashion.

One of the disadvantages of IF procedures is that the fluorescent stain will eventually fade. Conversely, IP staining provides a permanent record of the result and can also be easily adapted to detect more than one antigen. Comparative studies have indicated that IP assays are of equal, if not greater sensitivity and specificity than IF methods (Wicker, 1971; Hermmann et al., 1974; Hahon et al., 1975; Schmidt et al., 1978). Consequently, immunoperoxidase methods are gaining increased use for the localization of

viral antigens in both tissue culture (Benjamin and Ray, 1974; Desmond et al., 1979) and clinical specimens (Huang, 1975; Busachi et al., 1978; Kumanishi and Hirano, 1978). Therefore, it is obvious that the direct application of the PAP procedure in the investigation of clinical specimens can be used for the rapid diagnosis of virus mediated disorders. Much of the research carried out along this line has concentrated on the rapid diagnosis of herpes encephalitis (Benjamin and Ray, 1975; Merkel and Zimmer, 1981, 1982; White and Taxy, 1983). Similarly, research into the pathogenesis of other viral induced CNS disorders report that the PAP method is an effective method to study their pathological features (Merkel and Maibach, 1984; Bastien et al., 1984; Kristensson et al., 1984a).

XII. Prevention and Control of Arbovirus Disease

Control of arthropod-borne infections is an important problem today even though massive widespread epidemics or epizootics have all but been eliminated. Focal outbreaks of arbovirus infections are still known to occur in North America (Spence et al., 1977). These outbreaks have stimulated research into developing a number of alternative strategies with which to combat the spread of mosquito-borne encephalitides (McLintock, 1976; Monath, 1984).

One of the initial methods used to restrict the flow of arboviral agents was through the implementation of an equine vaccine (Burns, 1951; Binn et al., 1966; Monath and Trent, 1979). The use of such vaccines have markedly reduced the infection rate in the horse population. A search to produce a suitable human vaccine against WEE virus has met with moderate success (Bartelloni et al., 1971). Attenuated forms of WEE virus have been tested for

their efficacy as antigenic stimuli for vaccine production, but they have failed to gain widespread use (Olitsky et al., 1945; Dunayevich et al., 1961; Johnson, 1963). One of the reasons why these vaccines have not been readily accepted may be due to the difficulty in predicting the geographic focus of future epidemics.

Monath (1984) has outlined three methods which can be used to control the extent of arboviral encephalitides. Firstly, the essential features of the ecological cycle of the arbovirus should be determined. This means the identification of vectors, host species, and geographical distribution. With this knowledge firmly in hand, local health authorities can take measures to limit vector-host interaction. This may be achieved through chemical spraying or by using biological insect control methods to diminish the vector population. Secondly, sentinel flocks may be monitored during the vector breeding season in order to determine vector infection rates. This provides an early warning system signalling a potential epidemic situation. The third strategy available to inhibit arbovirus dissemination relies on the use of rapid diagnostic methods to identify the infecting virion. Early identification of the virus responsible for the encephalitis will allow public health officials to quickly instigate preventative measures to limit the scope of the infection. Of the methods currently available, immunofluorescent and immunoenzyme assays appear to be the most practical (Hildreth and Beaty, 1984; Hildreth et al., 1984; Monath et al., 1984).

Those victims unfortunate enough to become infected with WEE virus, presently receive only supportative treatment. However, immune sera provided early in the course of the disease may help in reducing the severity of the disorder (Howitt, 1932).

CHAPTER II

MATERIALS AND METHODS

I. Viruses

The test strain of Western equine encephalomyelitis virus was isolated from a horse infected during the 1965 epizootic in Alberta (Morgante et al., 1968). Since then, this strain (WEE 65-HO 49) has undergone eight mouse brain passages.

The Eastern equine encephalomyelitis virus, isolated from Culiseta melanura, was provided by Dr. P.H. Coleman, Centre for Disease Control, Atlanta, GA., U.S.A. This strain (AR 167) has undergone an unknown number of passages.

II. Propogation of WEE and EEE viruses

Several litters of 3 to 4 day old newborn Swiss albino mice (Western strain) were each inoculated intracerebrally (i.c.) with 0.02 ml of a 10 LD_{50} (lethal dose) of virus. All mice exhibiting signs of infection some 32-38 hours post-infection were collected and sacrificed. No dead animals were included. The brains of the infected mice were removed and ground to a 20% suspension in sterile Hank's Balanced Salt Solution (HBSS), pH 7.2, supplemented with 100 International Units (I.U.) of penicillin/ml, 100 micrograms of streptomycin sulphate/ml, and 15 I.U. of mycostatin/ml. Ten percent fetal calf was also added to this preparation. The brain suspensions were centrifuged at 7700 x g for one hour at 4-6°C. The supernatant fluid was subsequently aliquoted into small vials, sealed, frozen, and stored at -70°C.

III. Antigen Titration

Partially purified suspensions of both WEE and EEE viruses originally containing 100 LD_{50} of each virus were diluted ten-fold in HBSS supplemented with antibiotics. Eight suckling mice (3-4 day old), were inoculated i.c. with 0.02 ml of each dilution of virus. The mice were then kept under observation for signs of illness for a period of 14 days. The titre of virus giving a mortality of 50% (LD_{50}) was calculated according to the method of Reed and Muench (1938). WEE virus had an LD_{50} of 10^9, while EEE virus produced a titre of 10^{10}.

IV. Preparation of Control and Infected Tissues

Prior to inoculating suckling mice for the propogation of infected tissues, the titres of the infecting viruses were reassessed by the methods previously described. The adjusted LD_{50} of WEE virus and EEE virus were found to be $10^{8.5}$ and $10^{9.75}$ respectively. Thirty one, 3-4 day old suckling mice received i.c. injections of WEE virus, whereas twenty three mice were inoculated i.c. with EEE virus (see Table-1). Both groups were injected with 0.02 ml of a 100 LD_{50} of virus. An identical dose of WEE virus was given to five mice via an intraperitoneal (i.p.) and subcutaneous (s.c.) route. A total of twenty four mice were inoculated i.c. with diluent only (HBSS, pH 7.2, supplemented with antibiotics). Following careful observation, mice manifesting overt signs of encephalitis were sacrificed and the organs under investigation were removed. These tissues included brain, heart, lung, liver, kidneys, spleen, and intestines. All organs were removed aseptically and placed directly into 4% formaldehyde. The brain was not removed from the skull in order to minimize

Table-1. Schedule of inoculation and sacrifice of experimental animals.

Mouse No.	Inoculation Route	Virus Inoculated	Date of Harvest	Organs Harvested Brain	Viscera
1-24	i.c.	diluent	16/5/85	+	+
25-32	i.c.	WEE	23/5/85	+	+
33-37	i.p. & s.c.	WEE	23/5/85	+	+
38-55	i.c.	WEE	30/5/85	+	+
56-60	i.c.	WEE	30/5/85	+	-
61-77	i.c.	EEE	06/6/85	+	+
78-83	i.c.	EEE	06/6/85	+	-

the extent of mechanical trauma. Following a two hour fixation period, the brain was cut into halves to allow the fixative to fully penetrate into the tissue. The organs were fixed in formaldehyde for seven days, thereby completely inactivating the infecting virion and allowing complete fixation of the tissue. The organs were subsequently processed using standard histological techniques and ultimately embedded into paraffin blocks. Coronal sections of brain tissue, cut serially, were obtained for the investigation of the CNS. Where possible, parallel sections were stained with PAP, hematoxylin and eosin (H&E), and Nissl stains, to facilitate the localization of WEE viral antigen in neural tissue. Sections to be stained by PAP, H&E, and all sections obtained from visceral tissues, were cut at a thickness of 5-6u. Those sections to be stained with the Nissl stain were cut at 12-14u. Ultimately, several hundred sections, taken at various levels within the brain and visceral tissues were stained by the PAP technique. The intensity of the staining reaction was judged according to the following protocol:

0 -no evidence of specific staining.

\+ -rare isolated focus of infection.

++ -moderate staining of viral antigen.

+++ -extensive staining of viral antigen.

V. Preparation of Antisera

Rabbit anti-WEE virus hyperimmune sera was prepared by inoculating four albino New Zealand female rabbits, 10-12 weeks of age, with a 100 LD_{50} dilution of WEE virus brain suspension without Freund's adjuvant. A summary of the inoculation schedule is provided in Table-2.

Neutralization tests were carried out to determine the level of neutralizing antibodies in the rabbit anti-WEE virus sera. Nine litters consisting of eight suckling mice (4 day old), were inoculated intraperitoneally (i.p.) with ten-fold dilutions of WEE virus ranging from 10^{-1} to 10^{-9}, prepared according to the method of Hammon and Sather (1969). Equal volumes of hyperimmune sera were mixed with each virus dilution prior to inoculation. For the control mice, each virus dilution was mixed with normal serum. The normal mouse serum LD_{50} was 10^{7}, whereas the rabbit anti-WEE virus serum LD_{50} titre was 10^{0}. Therefore, the rabbit serum had a log neutralization index (LNI) of 7. This sera was provided as a gift from Dr. O. Morgante.

Mouse anti-WEE virus and mouse anti-EEE virus ascites fluid was kindly provided by Dr. N. Karabatsos, Centre for Disease Control, Reference Arbovirus Laboratory, Greely, Co., U.S.A. The mouse anti-WEE virus (Fleming strain) serum had the following titres, CF> 1:64, HI> 1:320, and NT> 2.5 (LNI). The mouse anti-EEE virus (strain NJ/60) serum contained antibodies at the following levels, CF> 1:64, HI> 1:320, and NI> 2.5 (LNI).

Other antisera used for staining in the PAP procedure were obtained from commercial sources. These included normal goat serum (Cedarlane), normal mouse serum (Cedarlane), goat anti-mouse IgG (Pel-Freeze), goat anti-rabbit IgG (Cedarlane, Dako), rabbit anti-mouse serum (Cedarlane), rabbit PAP complex (Cedarlane, Dako), and mouse PAP complex (Sternberger-Meyer).

Table-2. Inoculation of rabbits for the production of rabbit anti-WEE virus sera.

	Interscapular	Intraperitoneal
Initial Injection	1.0 ml	1.0 ml
1st week	1.0 ml	2.0 ml
2nd week	2.0 ml	2.0 ml
3rd week	2.0 ml	2.0 ml
Two week rest period.		
Booster dose	2.0 ml	2.0 ml
Rabbits bled out three months later.		

All antisera were diluted with 0.05M Tris-Hydrochloride buffer containing 0.85% sodium chloride. Tris-HCl buffer 0.5M, pH 7.6, was used to rinse the slides following incubation with the developing reagent. A standard 0.85% saline solution was used for all other wash cycles. A freshly prepared 30mg% solution of 3,4,3,4 tetraminobiphenyl hydrochloride (diaminobenzidine) containing 0.05% H_2O_2 was used as the substrate in the final reaction. All chemical reagents were purchased through the Sigma Chemical Co., St. Louis, U.S.A.

VI. Absorption of Antisera

An absorption procedure using normal mouse brain tissue obtained from three adult Swiss albino mice was carried out on the mouse anti-WEE virus, anti-EEE virus, and rabbit anti-WEE virus sera. The brains were aseptically removed and ground into a 50% suspension in phosphate buffered saline (PBS), pH 7.4 to 7.6. The suspension was washed a total of three times with the final wash in 0.05M Tris-HCl saline buffer supplemented with 1000 I.U. of penicillin G/ml and 100 I.U. of mycostatin/ml. An equal volume of the sera was added to the suspension and with frequent mixing was incubated at 4°C for 48 hrs. The mouse brain-antisera suspension was subsequently centrifuged at 7000 x g for 15 min. The supernatant fluid was aliquoted into vials and stored at -40°C.

VII. Protocol for Immunoperoxidase Staining

Initially, all sections were dried in a 56-60°C oven for 3-24 hours. The sections were subsequently deparaffinized through 2 changes of xylene and and a series of decreasing concentrations of alcohol baths. The sections were ultimately rehydrated in water for 5 minutes. In order to block endogeneous

peroxidase activity, the slides were incubated in a freshly prepared 10% hydrogen peroxide-methanol solution for 20 minutes, with constant mixing on a rotator. The tissues were quickly rinsed in distilled water and washed for 5 minutes in saline. All wash cycles required constant mixing. Upon completion of this step, the excess fluid was blot dried and diluted normal serum was added to the sections. These tissues were subsequently incubated in a humid staining box for 20 minutes at room temperature. Following this incubation period, the normal serum was removed and any excess fluid was blot dried. Properly diluted primary antisera, as determined by a checkerboard titration, was used to cover the sections for 48 hours at 4°C in a humid chamber. The sections were then washed in 3 changes of saline within a period of 10 minutes. Once again the tissues were blot dried and covered with diluted link reagent. The slides were returned to the humid chamber and incubated at room temperature for 30 minutes. The slides were eventually put through a second wash cycle, with 3 changes of saline in 10 minutes. The excesss fluid was drained and the diluted developing reagent was added to the tissues. These slides were incubated at 4°C for 30 minutes in a humid chamber. The final wash cycle required 2 changes in saline and a final 5 minute rinse in 0.5M Tris-HCl buffer. Freshly prepared diaminobenzidine (DAB) was used as the substrate for the colour reaction. The sections were exposed to DAB for 6-8 minutes, followed by a 5-10 minute wash cycle in running tap water. Mayer's hematoxylin was used to counterstain the tissue. The sections were then dehydrated in an increasing gradient of alcohol baths. Finally, the tissues were cleared in xylene, mounted in Diatex, and coverslipped.

VIII. Determination of Antisera Dilutions for PAP Staining

A checkerboard pattern, or box titration was used to determine the dilution of antisera providing optimal staining results. Dilutions of the three primary antisera were tested against doubling diltutions for both the link and developing reagent (see Table-3). The combination of the three immunological reagents that provided optimal staining results was used to stain all ensuing sections. Quality of staining was judged according to the intensity of the reaction, ability to detect weakly positive foci, cross-reactive potential, and the degree of non-specific background staining.

IX. Controls for Serological Specificity

Control slides were implemented into the staining protocol to assess the serological specificity of the antisera. The reagents used in the staining procedure may have unwittingly contributed to the background stain. Consequently, saline was substituted for the following reagents: the primary antisera, the link reagent and the developing reagent. In all three instances, sections were stained after one of the three immunological reagents had been substituted with saline, while the other two reagents were added according to the protocol. This allowed an assessment to be made regarding the degree of background staining contributed by the individual antisera. Various viral antigen controls were also incorporated into the PAP method to ascertain the degree of serological cross-reactivity. Mouse anti-WEE virus sera was used to stain tissue infected with WEE virus, EEE virus, and non-infected tissue. The sections containing WEE virus served as positive antigen controls, the non-infected tissues were the negative controls, and the EEE virus infected tissues served to monitor the cross-reactivity of the anti-WEE virus sera.

Table-3. Checkerboard titration of antisera.

Reagent	Antisera Dilutions					
1)Primary Antisera:						
Rabbit anti-WEE	1:100	1:500	1:1000	1:2000	1:4000	1:8000
Mouse anti-WEE	1:100	1:500	1:1000	1:2000	1:4000	1:8000
Mouse anti-EEE	1:100	1:500	1:1000	1:2000	1:4000	1:8000
2)Link Antisera:						
Goat anti-rabbit IgG			1:50	1:100	1:200	1:400
Rabbit anti-mouse (whole serum)			1:50	1:100	1:200	1:400
Goat anti-mouse IgG			1:50	1:100	1:200	1:400
3)Developing Antisera:						
Rabbit PAP Complex			1:100	1:200	1:400	1:800
Mouse PAP Complex			1:100	1:200	1:400	1:800

For the staining of tissue sections infected with WEE virus, both a rabbit and a mouse PAP system were utilized. The immunological reagents and their appropriate dilutions are described in Table-4.

X. Modifications of the PAP Procedure

i) Incubation Period:

Tissue sections were initially incubated with the primary antiserum for 2, 24, 48, and 72 hours. Similarly, a variety of incubation temperatures were attempted. The primary antisera were tested at 4, 22, and 37°C. The purpose of altering the duration and temperature of this incubation period was to assess their influence on the sensitivity of the assay. All incubations were carried out in a humid chamber. All sections were stained according to the method described and visualized by light microscopy. An assessment of the staining quality was made based upon the specificity and sensitivity of the results. Immunocytochemical staining of the remaining sections took place under those conditions providing optimal results.

ii) Trypsinization:

A 0.1% solution of trypsin (Bovine Pancreas Type III, Sigma Chemical Co.), was freshly prepared in phosphate buffered saline containing 0.1% calcium chloride, pH 7.8, adjusted with 0.1N sodium hydroxide. The trypsin solution was warmed in a 37°C water bath. Trypsinization was allowed to take place for 120, 60, 30, and 15 minutes respectively. Parallel non-trypsinized sections were incubated in warm PBS for identical time intervals. Tissue undergoing both treatments included sections attached to clean glass slides, as

Table-4. Dilutions of immunological reagents utilized in the PAP technique.

a) Rabbit PAP System:	
Primary antisera:	Rabbit anti-WEE virus, at 1:1000
Link antisera:	Goat anti-rabbit, at 1:50
Developing antisera:	Rabbit PAP Complex, at 1:200
b) Mouse PAP System:	
Primary antisera:	Mouse anti-WEE virus, at 1:1000
Link antisera:	Goat anti-mouse, at 1:200
Developing antisera:	Mouse PAP Complex, at 1:800

The blocking sera (normal goat serum) was diluted 1:25 with Tris buffer.

well as slides pre-coated with LePage's Bond Fast white resin glue (LePage's Ltd., Montreal, Canada). The glue was tested for its capacity to maintain adherence of sections to the slides during treatment with trypsin.

iii) Imidazole Treatment:

The final DAB substrate was modified by the addition of a 0.1% imidazole solution. The solution was prepared in a fashion similar to that described by de Jong et al., (1984). Staining was carried out as delineated in the procedure. Sections were stained for 6-8 minutes, followed by a rinse cycle. The sections were then dehydrated through a series of alcoholic baths and cleared in xylene. Ultimately, the sections were mounted with Diatex and coverslipped. The degree of background staining was judged according to the staining intensity of the neuropil.

iv) Comparison of Link Reagents:

Tissue sections incubated with mouse anti-WEE virus sera were treated with either goat anti-mouse IgG (diluted 1:200), or rabbit anti-mouse whole serum (diluted 1:100) as the link reagent. The immunological reagent providing the best results was used in all further staining reactions.

CHAPTER III

RESULTS

I. Gross and Histopathological Features

Suckling mice inoculated i.c. with WEE virus became ill some 34-38 hours post infection. These diseased animals presented with paralysis, convulsions, dehydration, and spasticity. Those mice infected with EEE virus became ill 32-34 hours after receiving the initial viral dose. These animals were more severely ill than the WEE virus infected mice, as they rapidly deteriorated following the onset of clinical symptoms of viral encephalitis. In both instances, the mice became moribund or died just prior to being sacrificed. Mice inoculated i.p and s.c. also demonstrated signs of illness at time of sacrifice. Control mice which received diluent only were sacrificed following a similar 32-38 hour time interval.

Removal of the organs from EEE and WEE virus infected mice revealed brains with markedly congested blood vessels. The hyperemic vessels were prominent on the surface of the brain, particularly over the cerebral hemispheres. Brain tissue of a soft consistency was also a frequent observation. The lungs of EEE infected mice appeared solidly hemorrhagic, and their livers were pale and reduced in size. All other organs appeared normal.

Microscopically, few of the classic signs indicating an encephalitic attack could be detected. Rare isolated foci of necrotic neurons appeared as the sole feature of a virus induced encephalitis. Degenerating neurons were typically represented by cells with pyknotic nuclei and increased cytoplasmic eosinophilia. Such cells exhibited shrinkage of their perikarya with consequent pericellular vacuolization of the neuropil. These alterations were most evident

in the hippocampus and pons. The EEE infected mice were more likely to exhibit these histologic features. Congested blood vessels were quite prominent throughout the CNS. Nevertheless, there was no evidence to suggest that proliferation of the vascular endothelium had occurred. The lack of an inflammatory cell infiltrate was the most notable omission from the pathological picture. Aggregations of inflamatory cells could not be discerned within the CNS and no evidence of perivascular cuffing was presented. Microglial nodules were also lacking in the mice infected with the pathogenic viral organisms. There was no demonstrable astroglial hypertrophy or proliferation.

Microscopic examination of the visceral organs failed to reveal any evidence of a pathological process. Only a low grade pneumonitis in the lungs and a moderate degree of vascular congestion in the liver was detected in a few animals.

II. Application of Immunoperoxidase Method

Preliminary application of the PAP staining method to brain tissue harvested from suckling mice infected with WEE virus revealed extensive non-specific background staining. Using rabbit anti-WEE virus sera as the primary reagent, it was found that tissue sections stained by immunoperoxidase yielded areas of both intense viral antigen staining, as well as diffuse colouration of the brain parenchyma. Regions demarcated as specific sites of viral replication appeared laden with a dark brown granular deposit. The neural matrix exhibited a diffuse "muddy-brown" pattern of staining. In an attempt to determine the source of the non-specific staining of the neuropil, saline was used as a substitute for each of the immunological reagents. Close inspection of saline substituted control sections indicated that the primary antiserum was

largely responsible for the high background component. In order to lessen the extent of the non-specific staining a number of interpolated experiments were carried out.

Initially, the rabbit anti-WEE virus sera was absorbed with mouse brain tissue. This produced a marked reduction in the level of background staining, although it was not completely eliminated. A box or checkerboard titration was also completed to determine the proper dilution of the immunological reagents. The staining results indicated that optimal staining of WEE viral antigen occurred when the antisera were used at the following dilutions: rabbit anti-WEE (R-WEE) virus antisera at 1:1000, goat anti-rabbit (GAR) antisera at 1:200 and rabbit-PAP (R-PAP) complex at 1:100. Under these conditions the virus populated areas were darkly stained with a moderate degree of background stain. Further dilutions of the primary antiserum led to progressively lighter staining of the regions containing viral antigen. These areas were golden-brown in colour, as opposed to the dark brown granular deposit observed in heavily infected regions. The end-point titre where staining of the viral antigen was eliminated occurred at a dilution of 1:4000. Sections taken from mouse brain tissue infected with diluent only showed a slight degree of background colouration when stained with R-WEE virus serum. On the other hand, the EEE virus infected tissues were shown to be cross-reactive with R-WEE virus serum at low dilutions. However, under optimal staining conditions the cross-reactive component disappeared with only residual background staining present.

The initial staining reactions were carried out with an incubation period of 48 hours at $4^{\circ}C$. Subsequent analysis where both time and temperature were manipulated proved that these conditions offered the best staining results. A very short incubation period of two hours produced weakly positive stained viral

antigen within the CNS. A 24 hour exposure limit provided a more intense level of staining. Focal colouration was seen extensively throughout the brain so that even weakly positive areas became evident. Following incubation periods of 48 and 72 hours, PAP stained sections for WEE viral antigen demonstrated richly stained areas with deposits at specific sites of virus replication. A widespread distribution pattern revealing numerous infected regions within the CNS could be discerned. Along with an increase in sensitivity attributed to a longer primary incubation step, there was also a concomitant increase in the level of non-specific staining.

A comparison of the staining quality of sections stained by the immunoperoxidase method was made following incubation with the primary reagent at the following temperatures: 4, 22 and 37°C. There appeared to be no significant difference in the quality of staining at any given temperature. All three temperatures produced similar results, however a slight advantage was achieved by incubating at 4°C. At this temperature the sera overlaying the section was not likely to evaporate as quickly, consequently this temperature was chosen for all future staining reactions.

A 2 hour pre-treatment with a 1% trypsin solution provided minimal improvement in the staining character. There was no apparent increase in the degree of antigenicity, yet a slight decrease in the level of diffuse staining of the neuropil could be appreciated. The resulting increase in contrast between specifically stained viral antigen and background stain was minimal. Incubation with trypsin for shorter periods of time produced similar results. One of the difficulties associated with the use of proteolytic agents was the loss of adherence of sections from the glass slides. This was particularly true for those sections exposed to trypsin for long periods of time. LePage's glue served as an

acceptable adhesive under these conditions. The resin, spread as a thin film on the glass slide, allowed the tissue to adhere to the slide and did not contribute to any background staining.

Sections stained with a DAB solution containing 0.1% imidazole proved unhelpful. The intensity of the specifically stained viral antigen was not increased nor was there a significant depreciation in the degree of non-specific staining. As a result, this treatment was abandoned in all further experiments.

An assessment was also made on the quality of the mouse anti-WEE virus ascites fluid as a primary reagent. As with the the R-WEE virus sera, the ascites fluid was absorbed with suckling mouse brain. The optimal dilutions of the sera required in the PAP protocol as determined by a checkerboard titration were as follows: mouse anti-WEE (M-WEE) virus ascites fluid diluted to 1:1500, goat anti-mouse (GAM) IgG antisera at 1:100, and mouse PAP (M-PAP) complex at 1:400. The application of M-WEE virus ascites fluid by immunoperoxidase techniques to sections containing viral antigen would yield areas of dark brown granular deposits at specific sites of virus localization. The tendency towards non-specific staining was also high. No staining of viral antigen was observed when the M-WEE virus sera was diluted to levels beyond 1:8000. Non infected tissues responded well to the M-WEE virus primary antisera with no apparent non-specific labelling of brain parenchyma. Sections containing EEE viral antigen would be cross-reactive with the M-WEE virus sera at low dilutions, but under optimal conditions or with very dilute primary antisera this cross-reactivity was eliminated. Extended wash cycles and increasing the duration of the treatment with hydrogen peroxide-methanol failed to reduce the intensity of the background stain.

During preliminary workup studies using M-WEE virus serum as the primary reagent, the link reagent used was rabbit anti-mouse (RAM) whole serum. Extensive background colour developed with the use of the RAM sera as the link reagent. A switch in reagents was attempted where goat anti-mouse (GAM) IgG antisera served as the link reagent. A significant reduction in the level of the non-specific staining was readily observed. Therefore, the GAM serum was used to stain the remaining sections.

III. Distribution of WEE Viral Antigen in the CNS

Staining of tissue sections taken from mice infected intracerebrally with WEE virus by the PAP procedure revealed a widespread distribution pattern of the viral antigen in the central nervous system (see Table-5). Those areas representing regions of viral replication occurred primarily as isolated foci of infection. However, on occasion confluent areas of staining indicating intense virus reproduction could be appreciated.

Serial sections taken from the anterior portion of the brain demonstrated specific localization of WEE virus within the olfactory region. A few well stained cells within the lamina plexiformis interna could be detected. However, more intense deposition of the reaction product was most often detected in the lateral and posterior portions of the anterior olfactory nuclei. The degree of infection in this region was quite variable, nevertheless specifically stained infected neurons were always present. In this region, the sub-ependymal ventricular area rarely demonstrated the presence of WEE viral antigen.

One of the regions of the CNS to demonstrate consistent and intense staining of viral antigen by the immunoperoxidase method was the sub-cortical region of the frontal lobes. Sections taken from the brains of a number of mice inoculated with WEE virus were always found to be reactive within this region when stained by the PAP method. Several groups of infected cells containing viral antigen could be seen scattered throughout the sub-cortical region of the cerebral cortex. Intensely stained neurons were often seen in the area cinguli, whereas only a rare infected cell could be visualized along the longitudinal cerebral fissure. No deposits of viral antigen were seen within the meninges.

Table-5. Distribution of WEE viral antigen in the CNS of mice inocuated intracerebrally.

Anatomical Site	Intensity of Reaction
Olfactory	+
Subcortical region	++
Basal Ganglia	+
Thalamic Nuclei	++
Hypothalamus	+
Hippocampus	++
Midbrain	+++
Pons	+++
Medulla oblongata	+++
Cerebellum	+

The frontal region, though a common site of virus localization, expressed a lower concentration of viral antigen than the parietal region. Typically, the tempo-occipital region and the area retrosplenialis were moderately affected. The area pyriformis of the cerebral cortex usually produced a minimal amount of evidence to suggest viral replication.

In the basal ganglia, the caudate nucleus and the putamen were infrequent sites of positive specific staining, although a few well dispersed neurons containing viral antigen could be appreciated within this region in mice with fulminant infections. The corpus callosum and fornix failed to support viral replication. The globus pallidus and grey stripes provided similar results indicating that the growth of the infecting virion was extremely limited in this region of the CNS. The amygdaloid nuclei were also negative. The periventricular region of the basal ganglia was one of the few areas to support viral replication. However, it was the septal and lenticular nuclei which contained the most intensely stained neural elements. Localization of WEE viral antigen in the sub-ependymal layer of the third ventricle continued to be a rare observation.

The thalamus of the suckling mouse appeared to be a preferred site for the reproduction of WEE virus. Many of the thalamic nuclei and various segments of the hypothalamus were susceptible to WEE virus invasion. Deposits of brown stained reaction product could be seen at the margin of the anterior thalamic nuclei. Moderate staining of the medial and lateral thalamic nuclei was also observed. The hypothalamus and the periventricular area of the thalamus frequently exhibited well stained neural elements. The ependymal cells lining the ventricles were also found to be capable of sustaining viral growth. The sub-fornical organ and the perifornical area of the thalamus were common sites

of virus invasion. Cells from the ventri-medial hypothalamus, the amygdaloid region and the caudate nuclei could on occcasion support a focus of infection. In cases of extensive viral replication, WEE viral antigen was also detected in the entopeduncular nuclei and the suprafascicular commisure posterior nuclei. Regardless of the degree of infection, the habenular nuclei represented an area of extensive viral replication. In contrast, the substantia nigra was spared from viral attack.

The hippocampal region of the CNS presented with numerous foci of infection. Although the anterior hippocampus appears to be insensitive to WEE virus, certain regions of the hippocampus, specifically the cells of the pyramidal layer, are likely to become infected. The hilus fascia dentate was also seen to contain antigenic markers to WEE virus. The stratum granulosum area dentate responded variably to infection with WEE virus. In more fulminant cases, several cells from this layer demonstrated the capacity to sustain viral particles. The stratum radiatum also exhibited a rare infected cell in severe cases while the stratum moleculare area dentate on occasion showed areas of small focal coolections of virus infected cells. The tegmental area also contained a significant concentration of antigen.

The midbrain represents an area of extensive viral growth following arbovirus infection with WEE virus. Neurons surrounding the cerebral aqueduct frequently demonstrated staining of WEE viral antigen. The lateral or medial geniculate bodies contain neurons immunoreactive with anti-WEE virus antibodies. The superior colliculi were less likely to be infiltrated with WEE virus. The nuclei loci ceruli and the ventral tegmental nuclei (Von Gudden) were uniquely insensitive to WEE virus while the surrounding neural tissue demonstrates a high degree of susceptibility. The substantia nigra was again

free of WEE virus. Isolated neurons in the pyramidal tract entrapped a few virus particles.

In the pons, several nuclear groups proved to be susceptible to WEE virus invasion. The most consistently stained areas occurred along the midline where the raphe dorsalis, raphe magnus, and raphe pallidus were moderately infected. The lateral and spinal vestibular nuclei presented with similar findings, whereas the medial vestibular nuclei allowed massive viral replication to take place. The periventricular grey area was a site exquisitely sensitive to WEE virus reproduction. The trapezoid nuclei and the dorsal aspect of the cochlear nuclei often could be seen as focal points of infection. The pontine nuclei, including the reticular and the ventral tegmenti nuclei, as well as the inferior colliculi demonstrated a moderate degree of variation in their response to WEE virus infection. Mice exhibiting a mild infectious process following i.c. inoculation of WEE virus showed no evidence of staining in these nuclear groups. However, those mice responding with a fulminant infection were likely to show a few immunoreactive neurons in this region. The nuclei parvocellularis and the motor nuclei of the trigeminal nerve were also seen as sites of viral replication.

In the cerebellum, the large Purkinje cells often demonstrated intense staining with M-WEE virus sera following the application of the PAP technique. Otherwise, the molecular layer and the granular layer were negative for viral antigen deposits. In a few instances, an isolated neuron in the nodular granular layer could be seen exhibiting evidence of cytoplasmic localization of WEE virus antigenic markers.

In the medulla oblongata, the substantia grisea centralis was the area demonstrating the greatest concentration of WEE viral antigen. Foci of infection occurred in the reticular formation and the hypoglossal nuclei. A few

positively stained cells could be seen in the inferior olives. The dorsal motor nuclei of the vagus were also infected with WEE virus. However, the spinal trigeminal nuclei, the nuclei ambiguus and the pyramidal tract of the medulla oblongata were negative for the presence of WEE viral antigen.

Immunoperoxidase staining of brain tissue taken from mice infected i.p and s.c. also indicated widespread localization of viral antigen. However, the extent of viral distribution was somewhat restricted as compared to mice infected via the intracerebral route. Viral antigen was rarely localized in the olfactory region and was not as widely distributed in the thalamic nuclei or in the hippocampus.

IV. Localization of WEE Viral Antigen Outside the CNS

Immunoperoxidase staining of coronal brain sections also revealed the presence of viral antigen in cranial ganglia. At the level of the optic chiasm, several cell bodies within the trigeminal ganglion were stained positive for WEE viral antigen. The surrounding satellite cells were apparently resistant to infection as no staining was detected. Cranial nerve trunks cut in cross-section were not stained by the PAP procedure. Neither the nerve fibres nor the Schwann cells gave any indication of containing viral particles.

Evidence indicating that viral replication had taken place in the eye of some of the infected animals was also observed. Cytoplasmic staining was seen in the ganglion cell layer of the retina.

The intracerebral, the intraperitoneal, and the subcutaneous infected mice all failed to produce reactive foci of infection in any of the visceral organs.

V. Localization of WEE Viral Antigen at the Cellular Level

Specifically stained neurons containing antigenic determinants to WEE virus, consistently demonstrated deposition of the reaction product within the cytoplasm and the dendritic and axonal processes. A few glial cells appeared to have a cytoplasmic border containing viral antigen. Cytoplasmic localization of WEE virus was also visualized in the ependymal cells lining the ventricular spaces and in the retinal cells. Intranuclear staining was never observed.

CHAPTER IV

DISCUSSION

The object of this investigation was to localize WEE viral antigen in infected suckling mice using the peroxidase-antiperoxidase technique. It was hoped that this study would provide important insights into the applicability of this immunohistochemical procedure for the rapid identification of viral agents responsible for encephalitic disorders, as well a means of studying the pathogenesis of viral encephalitides. Since WEE virus remains a significant cause of encephalitis in western Canada and the U.S.A. this viral agent was chosen for the present investigation. Suckling mice served as the host system for viral replication since these animals have shown a high degree of susceptibility to arboviral infection.

One of the difficulties associated with the application of immunohistochemical methodologies to tissue containing infectious agents, involves the potential hazard to personnel handling infected material. For this reason, a fixative such as formaldehyde which could inactivate the virion and provide adequate tissue fixation was utilized. Formaldehyde fixation also proved to be advantageous because viral antigenicity, essential for immunoperoxidase staining, was maintained. Even though earlier reports indicate that other fixatives such as Carnoy's fluid are most effective for the treatment of WEE virus infected tissue (Kundin and Liu, 1964; Albrecht, 1966), the data presented in this study suggests that formaldehyde fixation is equally effective and in fact offers an advantage because it is routinely employed in tissue processing.

In order to achieve satisfactory results, the conditions for the staining of viral markers in tissue sections must be pre-determined. These conditions

include the time and temperature of incubation with the various immunological reagents, the appropriate dilution of all antisera, and the serological specificity of the immunological reagents. Though many of these parameters have been determined and incorporated into standard PAP protocols, for diagnostic purposes it is imperative that all variables be standardized to peak efficiency if erroneous interpretation is to be avoided.

During the course of this investigation, it was observed that the duration and temperature of the initial incubation period with the primary antisera markedly affected the final staining results. A 24 to 48 hour incubation step at 4°C tended to provide the best results. This incubation period could be shortened to 2 hours, thus truly creating a rapid diagnostic system. However, the sensitivity of the assay would be compromised so that weakly infected sites may go undetected. If viral replication following initial infection proceeds at sufficiently high rates, then adequate numbers of viral particles would be produced early in the acute phase so as to be detectable by IP staining. Conversely, when the initial infecting dose is minimal or if the viral agent is slow growing, than several days may be required to achieve demonstrable levels of virions. Therefore, the point in the infection process at which the biopsy is taken may be critical in determining the final result.

For diagnostic purposes, the primary antisera must be of sufficiently high titre to allow the detection of low levels of viral antigen in tissue specimens. As well, non-specific or cross-reactive antibodies cannot be tolerated if closely related viral species are to be distinquished. A checkerboard titration serves as the most effective means of determining the appropriate dilution of the immunological reagents. A comparison of the two primary sera tested in this study reveals that M-WEE virus serum could be utilized in a more dilute form

than R-WEE virus serum. However, the neutralization tests had indicated that the R-WEE virus serum had a higher antibody titre than the M-WEE virus serum. Therefore, from the staining results it appears that the mouse anti-WEE virus antibodies must be of higher avidivity in order to account for the increased intensity of the final reaction product. It is this efficient binding capacity that allows these antibodies to couple to their antigenic counterparts, resulting in an intense staining pattern. The R-WEE virus antiserum likely contained a more heterogeneous population of antibodies, the majority of which were weakly binding. As a consequence, a more dilute solution of M-WEE virus serum could be used to achieve the same level of staining intensity observed with the R-WEE virus serum. One of the adavantages of using a more dilute primary reagent was that the non-specific component was also further diluted, thereby decreasing the degree of background staining.

As this investigation progressed, it became readily apparent that non-specific background staining was a problem. Preliminary stained sections expressed significant levels of non-specific staining of the neuropil. In an effort to eliminate or minimize the extent of this unwanted staining, a number of alternatives were investigated.

The most effective treatment to reduce background staining involved the absorption of the primary antisera with mouse brain tissue. This procedure removed many of the unwanted antibodies such as those directed against normal mouse brain tissue or non-viral proteins. The participation of these non-specific antibodies in the staining process had led to high levels of background colouration. Although the absorption procedure led to a significant dimunition in the degree of non-specificity, the background staining was not completely eliminated.

Increasing the length of the incubation period with the normal serum, in the hope of blocking any non-specific binding sites, showed little or no improvement in staining quality. Consequently, this procedure was discontinued. Similarly, a protocol utilizing extended wash cycles and hydrogen peroxide treatment was instituted in an effort to decrease non-specific staining. Once again, these changes in the methodology failed to provide favourable results.

Another alternative taken to reduce the intensity of the background stain entailed the use of the proteolytic agent trypsin. Several reports have indicated that digestive enzymes may reveal increased numbers of antigenic sites or destroy non-specific antibody binding sites (Curran and Gregory, 1975; Huang et al., 1976; Reading, 1977; Mepham et al., 1979). The end result is an increased signal to noise ratio or a more sensitive assay. With an increase in antigenicity, this would allow the use of higher dilutions of the primary reagent. Once again, this would assist in further diluting the non-specific component. Since the CNS contains a great deal of lipid, it is not surprising that proteolytic digestion only marginally improved the staining of virus infected brain tissue.

Recent reports in the literature have also indicated that a 0.1% solution of imidazole incorporated into the final chromogenic reagent will assist in decreasing non-specific staining (de Jong, 1984). The tissue enzyme catalase, which will breakdown hydrogen peroxide in the final PAP reaction, can contribute to the background non-specificity. Imidazole is an inhibitor of catalase, and will therefore reduce the level of this non-specificity. However, brain tissue has little endogeneous catalase activity, consequently treatment with this reagent failed to produce the desired results.

The link reagent was also found to contribute to the degree of background staining. The RAM sera derived against whole mouse serum contains

antibodies to all antigenic fractions found in mouse serum. Alternatively, the GAM sera was produced specifically against the mouse IgG fragment. Therefore, the GAM sera contained fewer non-specific antibodies which were capable of binding to the tissue and leading to increased levels of background colouration. These results serve to underscore the importance of utilizing specific, high titred, antisera in order to optimize staining results.

Suitable cross-reactive controls also played an important role in assessing the specificity of the procedure. Mouse brain tissue infected with EEE virus was observed to yield moderate degrees of positive staining with M-WEE virus sera. The common group antigenic markers that exist among alphaviruses are likely responsible for the production of cross-reactive antibodies which can lead to incorrect diagnosis of the etiological agent in viral encephalitis. Therefore, in order to differentiate between closely related species, type specific viral proteins should be utilized to elicit antibody production. The resulting antibody population would be heterogeneous in terms of avidivity, but antigenically specific. Ideally, monoclonal antibody preparations would be well suited for immunoperoxidase staining of infectious agents because of their unique specificity. The most recent indications suggest that monoclonal antibodies will function quite well as immunohistochemical reagents (Kristensson, et al., 1983). Such reports further emphasize the essential requirements of highy specific primary antisera for the diagnosis of distinct etiological agents by IP methods. Other valuable techniques such as column chromatography can be utilized to elute out any non-specific or cross-reactive antibodies. Once all the technical parameters have been standardized, it is then possible to apply the PAP technique to the diagnosis of viral infections of the CNS.

The results obtained in this study are consitent in many respects with the findings of previous reports investigating the pathogenesis of arboviral encephalitides. Firstly, EEE virus was found to be the more virulent of the two viral pathogens. Mice inoculated with EEE virus developed clinical symptoms of encephalitis 2-4 hours earlier than WEE virus infected mice. This is in agreement with the observations of Liu et al. (1970), who found EEE virus would produce a rapid disease with high viral titres in brain tissue. The highly virulent character of EEE virus was attributed to its ability to multiply at a faster rate than WEE virus. Various authors (Johnson, 1980; Janssen et al., 1984) have speculated that this increased susceptibility results because EEE virus is more efficient in the initial adsorption, penatration and uncoating processes required for viral replication. Rapid adsorption has been linked to the presence of numerous EEE virus receptors on the cell surface.

Perhaps virulence may also be a function of a more efficient replication mechanism as directed by the viral DNA. Exceedlingly efficient viral enzymes may allow for the rapid production of viral progeny during the exponential growth phase. Closely related but less virulent sub-species may contain greater numbers of nonsense codons arising due to genetic mutations leading to the formation of non-infectious virions.

Secondly, both EEE and WEE virus exhibited a high degree of neurotropism regardless of the route of inoculation. A few mice were inoculated via the intraperitoneal and subcutaneous route as a preliminary comparative study. Mice infected by these pathways also developed an acute encephalitic illness. Those animals inoculated i.c. were more severely ill at the time of sacrifice. This observation was substantiated by staining with the PAP technique which revealed intense staining and a widespread distribution of viral

antigen in the CNS. Mice receiving the virus suspension via i.p. and s.c. routes produced a comparatively restricted focus of infection. In the i.p. and s.c. infected group, the viral particles required an appreciable length of time to reach the CNS before viral replication could take place. In contrast, those particles placed directly into the brain could begin replication immediately. The delay noted in detecting viral progeny in the neural tissue of mice infected i.p. and s.c. has been associated with the time required to develop a sufficiently elevated viremia that would allow infection of the CNS (Wong, 1963; Harrison et al., 1980, 1982). Mesodermal tissues such as muscle, cartilage, and bone marrow which have been implicated as the primary sites of viral replication responsible for seeding of the bloodstream (Grimley and Friedman, 1970b), regrettably were not examined in this investigation. However, the absence of viral antigen in PAP stained sections taken from visceral organs clearly showed that these tissues would not likely be responsible for maintaining elevated viremic levels. The extent of viral replication in visceral tissues is also dependent upon the route of inoculation (Peck and Sabin, 1947; Albrecht, 1957b). But in this study neither the i.c. nor the i.p. or s.c. routes led to detectable levels of viral antigen in the peripheral tissues. It is believed that virus production did occur in many of these tissues, but that the levels of viral antigen fell below the sensitivity of the assay. Evidence supporting this has been offered by Liu et al. (1970) who also failed to detect viral antigen in visceral organs using immunofluorescent techniques. Nevertheless, they were capable of demonstrating virus production in these tissues through routine titrations of infectivity. Therefore, it seems feasible that viral replication occurred, but at much lower rates than those present in the brain. Subsequent analyses of viral pathogenesis

would be wise to monitor virus production in both muscle tissue and fibroblasts which act as key repositories of viral agents in peripheral tissues.

A third observation frequently cited in studies of arboviral pathogenesis (Aquilar, 1970; Mims et al., 1973; Janssen et al., 1984) was the absence of the histopathological features of an acute encephalitis. An occasional necrotic neuron provided the only evidence of a pathological process. These lesions although noted in WEE infected brain tissue, were much more prominent in EEE virus infected animals. Microglial proliferation and an inflammatory cell infiltrate were also absent from the histopathological picture. The minimal evidence indicating morphological and pathological change in the brains of mice experimentally infected with WEE virus was an unexpected observation. The intensity of the final stained product also suggested that an abundance of virus progeny had been produced. These two factors, however do not necessitate lysis of the host cell, but may merely reflect the efficiency of the cellular system as a virus producing factory. If the virus particles remained sequestered within the cell until late in the infectious process, this delay could account for the lack of histopathological changes. Since the viral proteins which incite the immune response would only become evident late in the infection, then the immune system may not have sufficient time to mobilize the various defense mechanisms that ultimately contribute to the pathological process. A similar argument has been put forward proposing that fulminant viral infections can develop and not allow the immune mechanism enough time to mount a visible response (Eldadah and Nathanson, 1967; Albrecht, 1968; Liu et al., 1970). A variety of sources can contribute to the development of fulminant infections. A high initial viral dose or direct inoculation of the pathogen into the CNS will often lead to agressive viral encephalitides. Similarly, an immunocompromised host such as a

developmentally immature animal will be acutely sensitive to a raging viral infection. In this investigation, both the immaturity of the host (1-3 day old suckling mice) and the route of inoculation (i.c.) could be seen as factors contributing to the limited histopathological changes observed. However, mice inoculated i.p. and s.c. also failed to reveal substantial changes in the neural tissue. As well, adult mice have been shown to produce minimal histopathological lesions following viral encephalitis (Liu et al., 1970; Janssen et al., 1984). Therefore, it seems that other factors are more important in determining the final pathological picture.

In terms of localization, the PAP procedure revealed the midbrain, the pons, and the medulla to be the areas containing the most extensive distribution of WEE viral antigen in the CNS. The sub-cortical region, especially the area cinguli, as well as the periventricular area of the thalamus, the hippocampus and cerebellum were often laden with viral antigen. These results are comparable to previous reports in the literature (Johnson, 1968; Eldadah and Nathanson, 1967; Liu et al., 1970; Mims et al., 1973) which have indicated that the cerebral cortex, portions of the hippocampus, and the Purkinje cells of the cerebellum often contain viral antigens following arbovirus infection. The study differs from these early investigations in that the basal ganglia and the corpus striatum which have been previously implicated as primary sites of attack during arboviral invasion were not major sites of viral antigen deposition.

Whether anatomical connections, selective vulnerability of neural cells, or specific virus strains are responsible for the distribution pattern of the pathogen in the CNS is difficult to assess. Future investigations concerned with the movement of viral particles within the neuropil making use of stereotaxic inoculation procedures would allow for precise localization of the infecting

dose, and analysis of tissue taken at various stages in the infection process in order to reveal the migration route of the virions within the CNS. Such studies would indicate if anatomical communications play a central role in determining virus transport or whether viruses are propogated to intrinsically susceptible neural sub-populations. Intrinsic susceptibility of cells has been associated with the complex function of neuronal nuclei, which manifests itself through the production of a variety of cellular proteins many of which act as viral receptors (Johnson, 1980).

WEE virus was also detected in the ganglion cell layer of the retina. Mims et al. (1973) had reported immunofluorescent staining of group-A arboviral antigens in the inner and outer nuclear layers of the retina. Such evidence along with the localization of WEE viral antigen in cranial ganglia is strongly suggestive of centrifugal spread. This form of centrifugal spread is common in the end stages of infection with arboviral agents (Eldadah and Nathanson, 1967). This data would suggest that arboviruses such as WEE virus are capable of being transported along a neural route. However, it is not clearly understood how viruses are propagated along nerve fibres. Since axons contain RER which is essential for virus replication, an axoplasmic transportation route has been postulated (Johnson, 1982). Immunoperoxidase staining was not seen in endoneural cells, but rather in the perikarya of infected ganglion cells. Therefore, the possibility exists that WEE virus replicates in the cytoplasm of ganglion cells and is conducted via the axoplasm.

WEE virus was found strictly in the cytoplasm of all infected cellular elements. There was no evidence to support intranuclear localization. These results confirm the fact that WEE virus replicates within the cytoplasm of host cells. Other studies have demonstrated arboviral antigens within the nucleus,

but this likely represents late stage degenerative changes such as a breakdown in the nuclear membrane (Hamashima et al., 1959).

Neurons were the cell type most often infected. Even though many of these cells were intensely stained indicating profuse viral production, very few neurons exhibited morphological change. Early degenerative features of WEE include the formation of cytopathic vacuoles, but their significance is poorly understood (Wolinsky and Johnson, 1980). The development of such vacuolar lesions along with focal areas of demyelinization and the minimal structural alterations seen in the host cell is consistent with many of the features associated with "slow viral infections". The possibility that WEE virus might, under some circumstances, behave as a "slow virus" is an intriquing one.

The PAP technique also demonstrated viral antigen within the dendrites and axons of neurons. This observation is of particular importance because it suggests an obvious cell to cell transfer mechanism. Cellular localization of viral particles using E.M. and immunoperoxidase methodologies would again be very useful in determining whether this is a viable pathway for the transportation of viruses within the brain. If viruses do travel by this system, then viral replication must proceed expidiously and the transport system must be extremely efficient in order to account for the widespread distribution of virus in the CNS.

Specific staining of viral antigen was also detected in the ependymal cells lining the ventricles. However, no deposition of reaction product was seen in the epithelial cells of the choroid plexus. Since these two regions are frequently the site of viral agents (Hamashima et al., 1959; Kristensson et al., 1983), and the two structures lie in close association, then the migration of virus particles from the vascular choroid through the ependyma and into the

CNS has been postulated (Johnson and Mims, 1968). The lack of immunoreativity in the choroid would suggest that the virus is not capable of moving from the CNS to the peripheral blood via this barrier. However, it may be that the virus simply has not had sufficient time to replicate to detectable levels in the epithelial cells. This does not appear to be the case as the intensity of the PAP reaction in the surrounding ependymal cells supports extensive viral replication in this area.

One of the unique observations of this investigation was the apparent lack of involvement of the glial cells. Only in rare instances was specific staining of glial elements demonstrated. It would not be unexpected to find a few microglial macrophages which have phagocytized virus particles. Since glial cells typically respond late in the infection process, a fulminant infection requiring only 32-38 hours to reach a fatal outcome may not allow large numbers of glial cells to become activated to the viral infection. It may also be argued that the microglial cells are still functionally immature, and therefore incapable of responding to a viral attack.

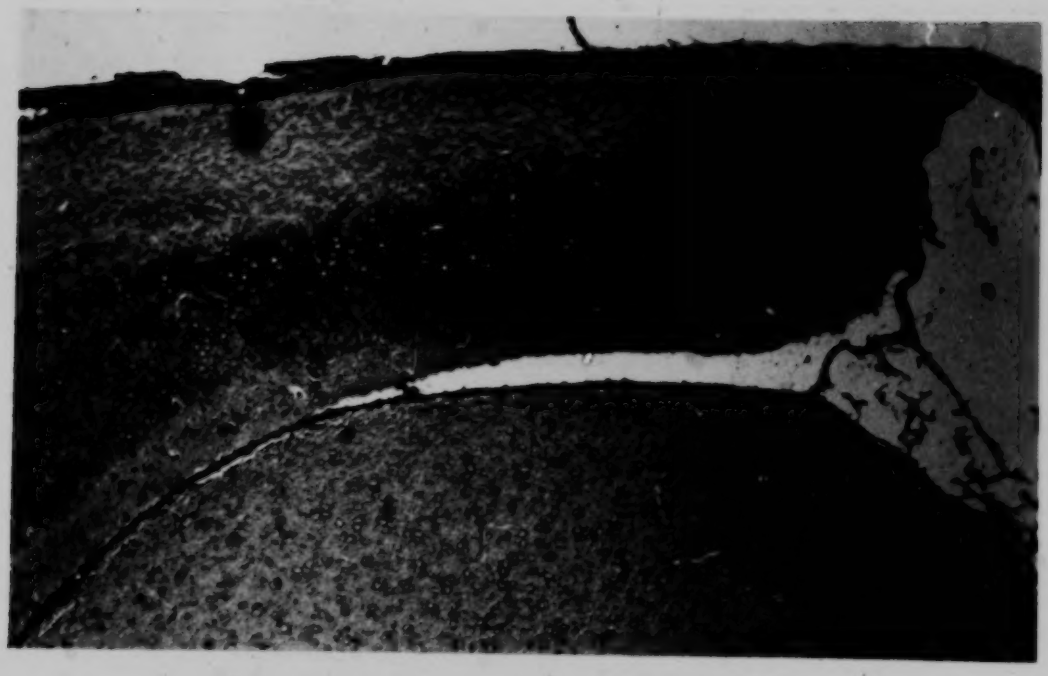

Figure 1 Immunoperoxidase staining in the sub-cortical region of formalin fixed, paraffin embedded mouse brain tissue infected intracerebrally. X 100.

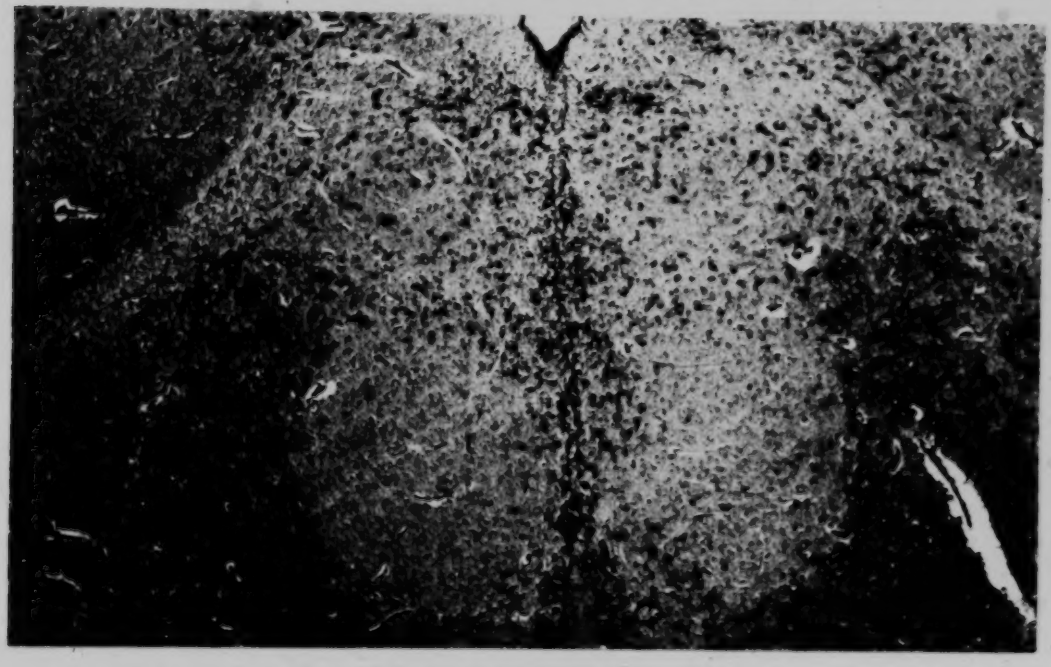

Figure 2 Extensive PAP staining of WEE viral antigen in the midbrain with the absence of staining on the loci ceruli and ventral tegmental nuclei. X 100.

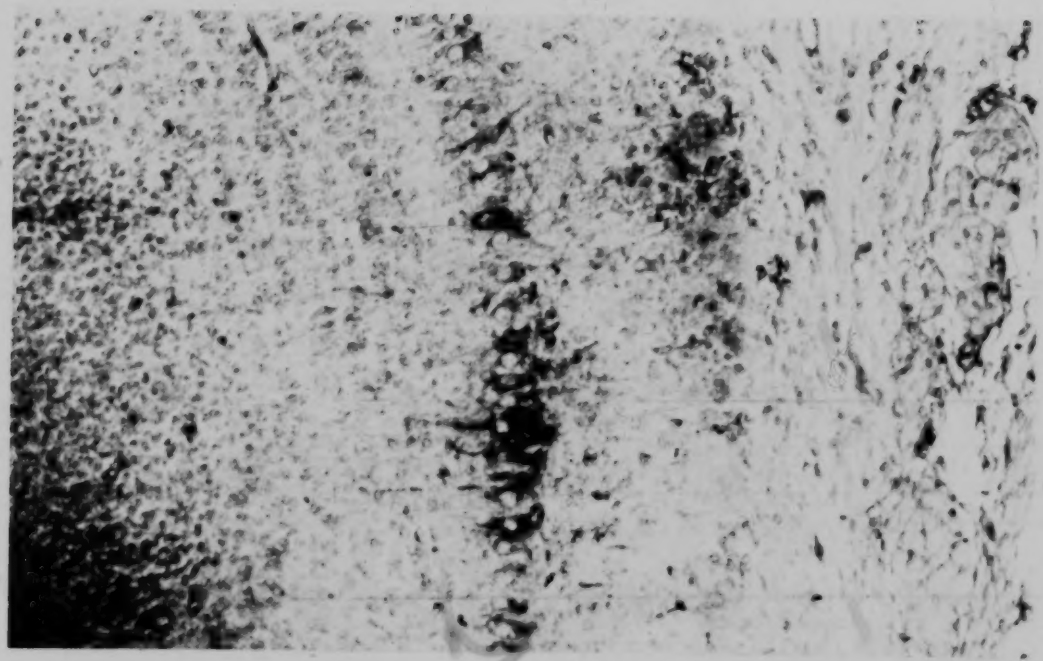

Figure 3 WEE viral antigen localized in lamina plexiformis interna cell layer of the olfactory region. PAP X 100.

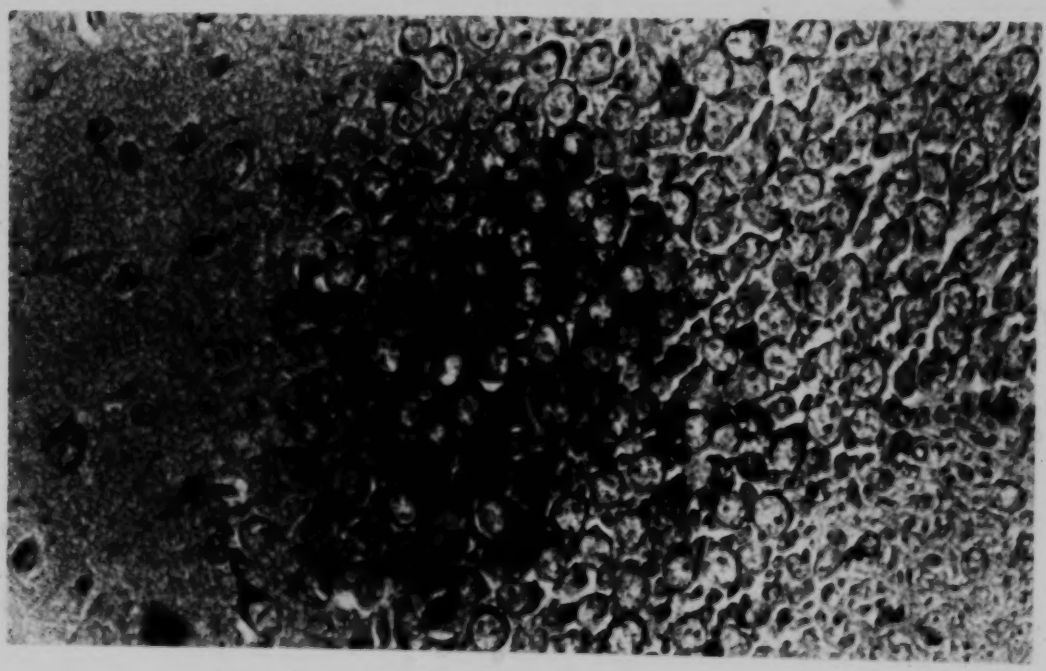

Figure 4 Isolated focus of WEE viral antigen as detected by immunoperoxidase staining in the pyramidal cell layer of the hippocampus. PAP X250.

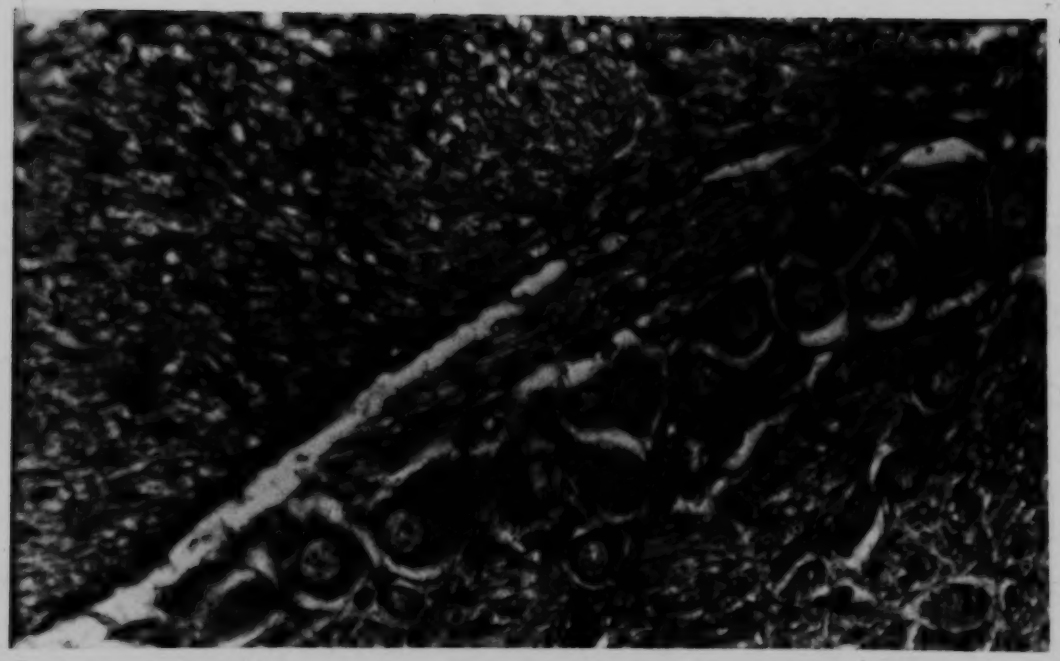

Figure 5 WEE viral antigen stained by immunoperoxidase localized within the perikaryon of cells of the trigeminal ganglion. X 250.

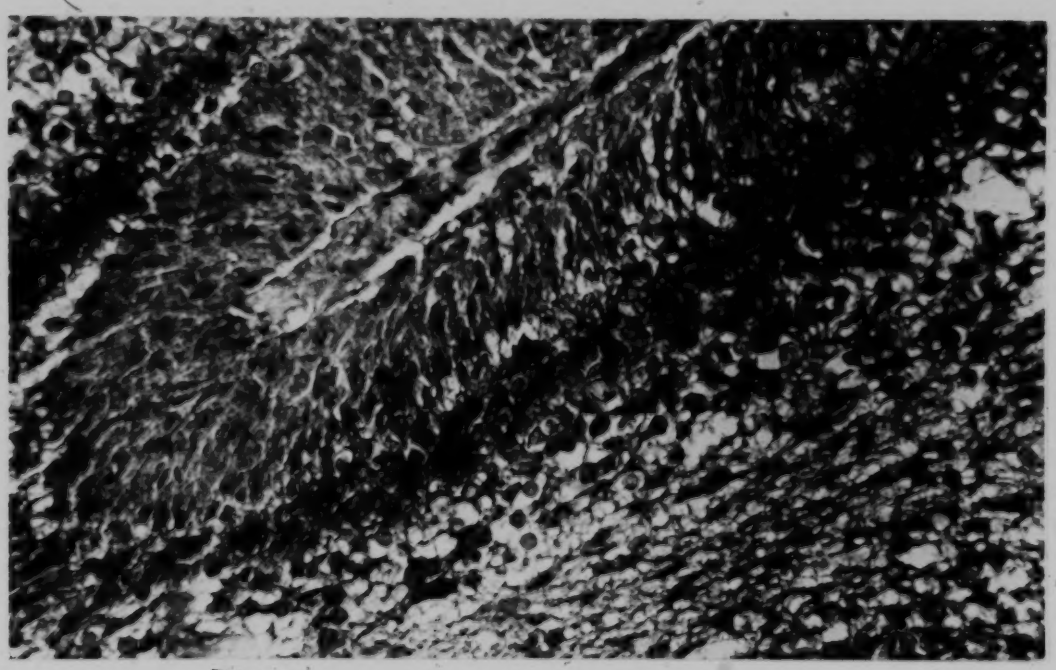

Figure 6 Darkly stained reaction product within the cytoplasm of the Purkinje cells of the cerebellum indicates the presence of WEE viral antigen. PAP X 250.

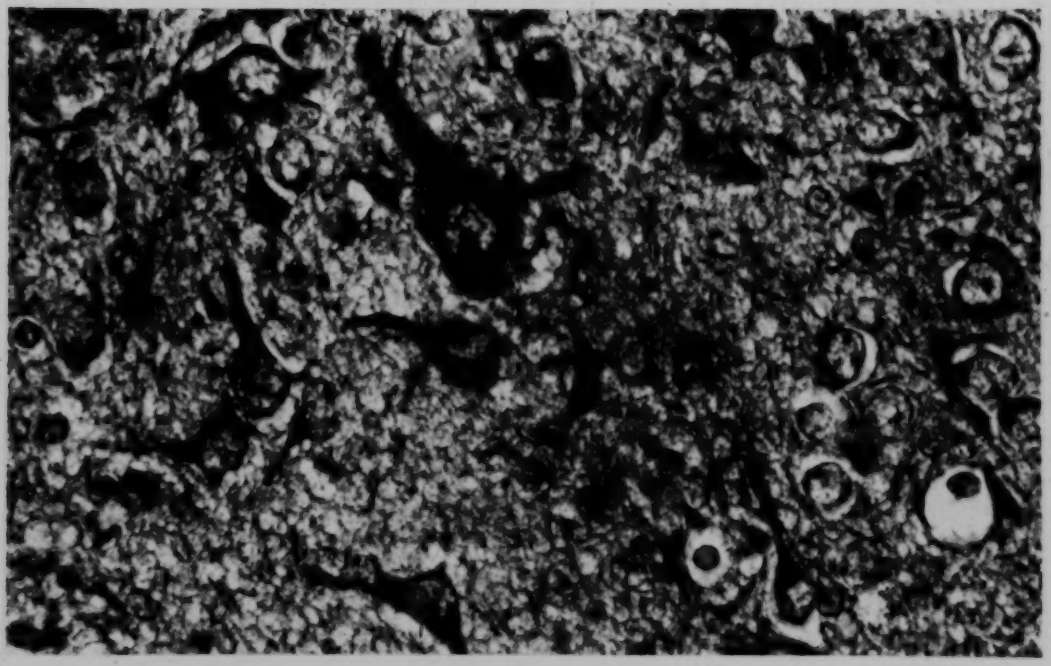

Figure 7 Immunoreactive neuron in the inferior colliculus, stained by the peroxidase-antiperoxidase technique. X 400.

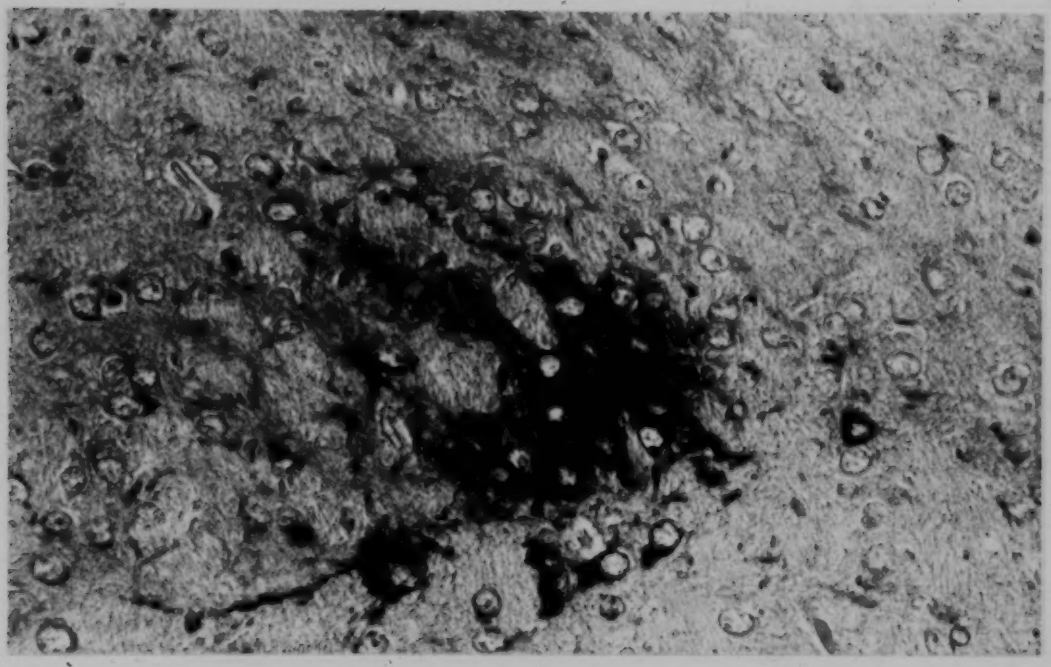

Figure 8 Positively stained thalamic nuclei demonstrated by immunoperoxidase indicates replication of WEE virus. X 250.

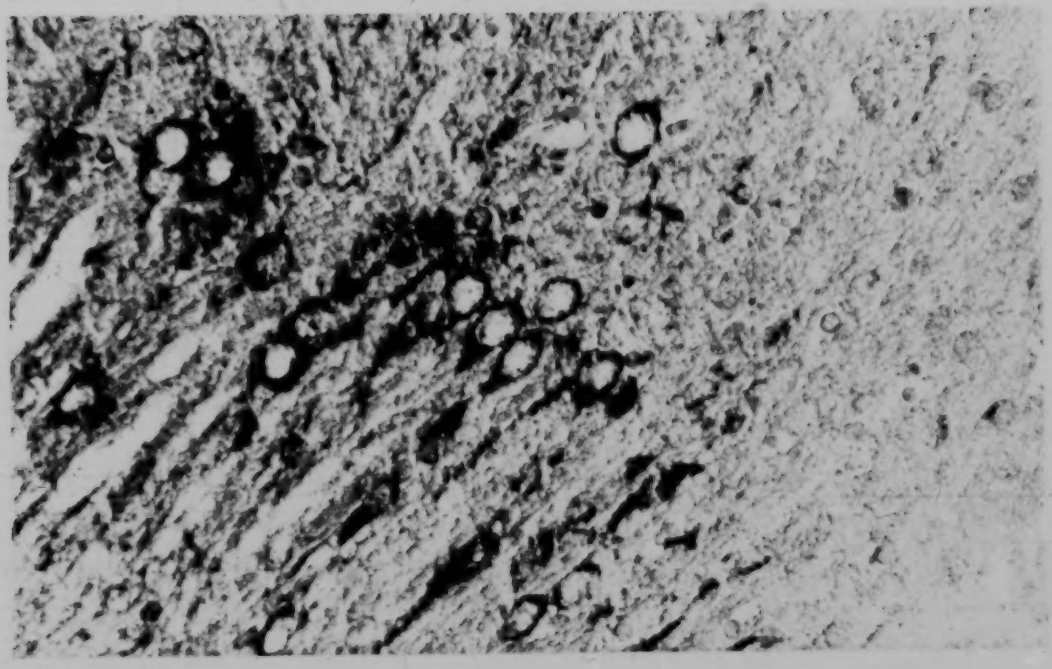

Figure 9 Cytoplasm and cell processes of neurons from the temporal region of the cerebral cortex reactive by immunoperoxidase. X 250.

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